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(56) References cited:
WO-A-88/00205 **WO-A-89/09788**

- **Lyons et al., 1989, Proc.Nat.Acad.Sci.USA, 86, p. 4554-4558**
- **CELL, vo. 51, 4. Dezember 1987, Cambridge, NA US pages 861-867; D.L. WEEKS et al: "A Maternal mRNA localized to the vegetal hemisphere in xenopus eggs codes for a growth factor related to TGF-beta".**
- **EMBO JOURNAL, vol. 8, no. 4 April 1989, EYNSHAM, Oxford GB pages 1057-1065; LESLIE DALE et al: "Developmental expression of the protein product of Vgl, a localized maternal mRNA in the frog Xenopus laevis"**
- **SCIENCE, vo. 242, 1988, LANCASTER, PA US pages 1528-1534; J.M. WOZNEY et al: "Novel regulators of bone formation: molecular clones and activities"**

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The file contains technical information submitted after the application was filed and not included in this specification

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Description

BACKGROUND OF THE INVENTION

The present invention relates to a DNA containing a DNA segment coding for a Xenopus laevis bone morphogenetic protein analogous to a bone morphogenetic protein (hereinafter referred to as BMP), in particular a precursor protein (or a precursor polypeptide) and a mature protein (or a mature polypeptide) of the Xenopus laevis BMP, and a method for preparing the precursor protein and the mature protein.

In this specification, the term "precursor protein" includes a protein which includes an amino acid sequence of a mature peptide Xenopus Laevis BMP and has all or a portion of an amino acid sequence coded with a Xenopus laevis BMP DNA segment at the N-terminus, the C-terminus or both termini thereof.

Recently, it has been revealed that transforming growth factor-beta (TGF-beta, TGF-β) having a bone morphogenetic activity not only controls cell proliferation, but also has various biological activities such as control of cell differentiation. In particular, the bone morphogenesis-promoting activity of TGF-β has been noted, and attempts have been made to use TGF for treatment of fractures and osteoporosis, making use of the cartilage-bone induction activity thereof [M. Noda et al., J. Endocrinology 124, 2991-2994 (1989); M. E. Joyce et al., J. Bone Mineral Res. 4, S-259 (1989); and S. M. Seyedin et al., J. Biol. Chem. 281, 5693-5695 (1986)]. More recently, however, four kinds of bone morphogenetic proteins (BMPs) which are different from one another in molecular structure have been identified as a factor promoting morphogenesis of bones and cartilages. Of these four kinds, human BMP-1, human BMP-2A, human BMP-2B and human BMP-3 are novel peptides, though they are very similar in structure to TGF-β, and there has been a report that they induce morphogenesis of bones and cartilages when subcutaneously or intramuscularly implanted in animals [J. M. Wozney et al., Science 242, 1528-1534 (1988)].

The above peptides having bone morphogenetic activity are isolated and purified from bones in which the peptides are considered to be localized, or from human osteosarcoma cells (U2-OS) which are thought to produce the peptides. However, such a method has problems because the procedure is complicated and the desired peptides are obtained only in small amounts.

SUMMARY OF THE INVENTION

Important contributions will be made to future studies and medical treatment, if a similar peptide having the bone morphogenetic activity can be collected from Xenopus laevis and further prepared by recombinant technique. As a result, the following information was obtained, thus arriving at the present invention.

Namely, the present inventors first succeeded in cloning five kinds of DNA coding for BMP-2A and related DNAs (Xenopus laevis BMPs) and subsequently three kinds of complementary DNAs, eight kinds of DNAs in total, by using a complementary DNA of a rat inhibin βA chain equally belonging to the TGF-β family as a probe. Further, the present inventors identified portions of the bases of the DNAs, clarified the amino acid sequences (see formulae (I), (II), (III), (IV) and (V) of Fig. 3 and formulae (VI), (VII) and (VIII) of Fig. 4) of the Xenopus laevis BMPs (referred to as B9, M3, C4, A4, A5, Xbr22, Xbr23 and Xbr41), and succeeded in pioneering their mass production by recombinant technique.

In accordance with the present invention, there are provided (1) a Xenopus laevis BMP,

wherein said protein is a mature protein containing an amino acid sequence having an amino acid sequence represented by Nos. 15 to 130 of formula (I) shown in Fig. 3, an amino acid sequence represented by Nos. 14 to 127 of formula (II) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 63 of formula (IV) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 65 of formula (V) shown in Fig. 3, an amino acid sequence represented by Nos. 282 to 398 or Nos. 298 to 398 of formula (VI) shown in Fig. 4, or an amino acid sequence represented by Nos. 328 to 426 of formula (VIII) shown in Fig. 4, and
wherein said protein is a precursor protein containing an amino acid sequence having an amino acid sequence represented by formula (I), (II), (IV) or (V) shown in Fig. 3, or formula (VI) or (VIII) shown in Fig. 4; (2) a DNA comprising a DNA segment coding for the Xenopus laevis BMP as defined in (1) and a DNA comprising a DNA segment coding for the Xenopus laevis BMP wherein said DNA segment comprises a nucleotide sequence corresponding to the nucleotide sequence represented by formula (1), (2), (3), (4), (5), (6), (7) or (8) shown in Fig. 2; (3) a non-human transformant bearing the DNA containing the DNA segment coding for the Xenopus laevis BMP as defined in (2) which is not Xenopus laevis ; (4) a method for preparing the Xenopus laevis BMP which comprises culturing the non-human transformant described in (3), producing and accumulating a protein in a culture and collecting the protein thus obtained. (5) a composition for therapy of fracture or osteoporosis containing the Xenopus laevis BMP defined in (1); and (6) a method for preparing the composition as defined in (5).

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows simplified restriction enzyme maps of DNA sequences containing Xenopus laevis BMP precursors or mature peptide DNA segments;

Figs. 2(1) to 2(8) show nucleotide sequences of the DNA segments of Xenopus laevis BMPs, B9, M3, C4, A4, A5, BMP-2A, BMP-2B and Vgr-1, respectively, and the amino acid sequences deduced therefrom;

Fig. 3 shows amino acid sequences of the Xenopus laevis BMPs deduced from the nucleotide sequences of the DNA segments shown in Figs. 2(1) to 2(5), comparing them with the amino acid sequences of known proteins having a bone morphogenetic activity; and

Fig. 4 shows amino acid sequences of the Xenopus laevis BMPs deduced from the nucleotide sequences of the cDNA segments shown in Figs. 2(6) to 2(8).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The mature Xenopus laevis BMP of C4, one of the Xenopus laevis BMPs, of the present invention, which has a relationship to TGF-β and is a peptide consisting of 98 or 114 amino acid residues, has an amino acid sequence represented by Nos. 6 to 119 or Nos. 22 to 119 of formula (III) shown in Fig. 3. The molecular weight thereof is calculated at about 25,000, excepting sugar chains, when a dimer is formed.

The amino acid sequence of this peptide is different from that reported by Wozney et al. in 3 or 4 amino acid residues per molecule.

Fig. 3 shows amino acid sequences of five kinds of novel Xenopus laevis BMPs obtained in the present invention, comparing them with the amino acid sequences of known proteins having a bone morphogenetic activity. In these amino acid sequences, the same amino acid residue as with βA is represented by ".", and an amino acid residue different from that of βA is represented by one letter symbol based on βA. CONSENSUS shown in Fig. 3 indicates amino acid residues common to all the BMPs shown in Fig. 3. The illustration of CONSENSUS results in introduction of gaps "-" in the formulae in Fig. 3. Accordingly, the number representing the precursor and mature protein portions is counted excluding these lacking portions.

Fig. 4 shows amino acid sequences of three kinds of novel Xenopus laevis BMPs deduced from cDNAS, subsequently discovered by the present inventors.

For DNA sequences, the DNA segments coding for the Xenopus laevis BMPs of the present invention correspond to the nucleotide sequences of formulae (1) to (8) (corresponding to B9, M3, C4, A4, A5, Xbr22, Xbr23 and Xbr41, respectively) shown in Fig. 2 or are portions thereof. Any functional portion can be used so long as bone morphogenetic activity is not lost. Wozney et al. reports the amino acid sequences, but does not elucidate the nucleotide sequences. As used herein the term correspond permits conservative additions, deletions and substitutions. Preferably, the DNA segments coding for the BMPs of the present invention have the nucleotide sequences of formulae (1) to (8).

With respect to the portion relating to the mature BMPs [the amino acid sequence represented by Nos. 15 to 130 of formula (I) shown in Fig. 3, the amino acid sequence represented by Nos. 14 to 127 of formula (II) shown in Fig. 3, the amino acid sequence represented by Nos. 6 to 119 or Nos. 22 to 119 of formula (III) shown in Fig. 3, the amino acid sequence represented by Nos. 6 to 63 of formula (IV) shown in Fig. 3, the amino acid sequence represented by Nos. 6 to 65 of formula (V) shown in Fig. 3, the amino acid sequence represented by Nos. 282 to 398 or Nos. 298 to 398 of formula (VI) shown in Fig. 4, the amino acid sequence represented by Nos. 288 to 401 or Nos. 304 to 401 of formula (VII) shown in Fig. 4, or the amino acid sequence represented by Nos. 328 to 426 of formula (VIII) shown in Fig. 4], the DNA sequences of the present invention differ from the DNA sequence of TGF-β, and therefore are novel.

As the DNA sequences coding for the BMP mature peptides of the present invention, any DNA sequences may be used as long as they contain nucleotide sequences coding for the amino acid sequences of the BMP mature peptides. For example, DNA sequences corresponding to the nucleotide sequences represented by formulae (1) to (8) or portions thereof are preferably used. More preferably the DNA sequences contain the nucleotide sequences represented by formulae (1) to (8).

The nucleotide sequences represented by formulae (1) to (8) are the Xenopus laevis BMP DNA sequences obtained in the present invention. Examples of the nucleotides coding for the Xenopus laevis BMP amino acid sequences represented by formulae (I) to (VIII) include Nos. 693 to 1040 of formula (1), Nos. 134 to 475 of formula (2), Nos. 435 to 728 of formula (3), Nos. 183 to 356 of formula (4), Nos. 149 to 328 of formula (5), Nos. 249 to 1442 of formula (6), Nos. 104 to 1306 of formula (7) and Nos. 86 to 1363 of formula (8).

An expression vector having the DNA sequence containing the nucleotide sequence coding for the BMP of the present invention can be prepared, for example, by the following process:

(a) Messenger RNA (MRNA) is isolated from BMP-producing cells.

(b) Single stranded complementary DNA (cDNA) is synthesized from the mRNA, followed by synthesis of double

stranded DNA.

(c) The complementary DNA is introduced in a cloning vector such as a phage or a plasmid.

(d) Host cells are transformed with the recombinant phage or plasmid thus obtained.

(e) After cultivation of the transformant thus obtained, the plasmid or the phage containing the desired DNA is isolated from the transformant by an appropriate method such as hybridization with a DNA probe coding for a portion of the BMP or immunoassay using an anti-BMP antibody.

(f) The desired cloned DNA sequence is cut out from the recombinant DNA.

(g) The cloned DNA sequence or a portion thereof is ligated downstream from a promoter in the expression vector.

The mRNAs coding for the BMPs can be obtained from various BMP-producing cells such as ROS cells.

Methods for preparing the mRNAs from the BMP-producing cells include the guanidine thiocyanate method [J. M. Chirgwin et al., Bio-chemistry 18, 5294 (1979)].

Using the MRNA thus obtained as a template, cDNA is synthesized by use of reverse transcriptase, for example, in accordance with the method of H. Okayama et al. [Molecular and Cellular Biology 2, 161 (1979); ibid. 3, 280 (1983)]. The cDNA thus obtained is introduced into the plasmid.

The plasmids into which the cDNA is introduced include, for example, pBR322 [Gene 2, 95 (1977)], pBR325 [Gene 4, 121 (1978)], pUC12 [Gene 19, 259 (1982)] and pUC13 [Gene 19, 259 (1982)], each derived from Escherichia coli, and pUB110 derived from Bacillus subtilis [Biochemical and Biophysical Research Communication 112, 678 (1983)]. However, any other plasmids can be used as long as they are replicable and growable in the host cells. Examples of the phage vectors into which the cDNA may be introduced include λgt11 [R. Young and R. Davis, Proc. Natl. Acad. Sci. U.S.A. 80, 1194 (1983)]. However, any other phage vectors can be used as long as they are growable in the host cells.

Methods for introducing the cDNA in the plasmid include, for example, the method described in T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, p.239 (1982). Methods for introducing the cDNA in the phage vector include, for example, the method of T. V. Hyunh et al. [DNA Cloning, A Practical Approach 1, 49 (1985)].

The plasmid thus obtained is introduced into the appropriate host cell such as Escherichia and Bacillus.

Examples of Escherichia described above include Escherichia coli K12DH1 [Proc. Natl. Acad. Sci. U.S.A. 60, 160 (1968)], M103 [Nucleic Acids Research 9, 309 (1981)], JA221 [Journal of Molecular Biology 120, 517 (1978)], HB101 [Journal of Molecular Biology 41, 459 (1969)] and C600 [Genetics 39, 440 (1954)].

Examples of Bacillus described above include Bacillus subtilis MI114 [Gene 24, 255 (1983)] and 207-21 [Journal of Biochemistry 95, 87 (1984)].

Methods for transforming the host cell with the plasmid include, for example, the calcium chloride method or the calcium chloride/rubidium chloride method described in T. Maniatis et al., Molecular Cloning, Cold Spring harbor Laboratory, p.249 (1982).

When the phage vector is used, for example, the phage vector can be transduced into multiplied Escherichia coli, using the in vitro packaging method.

Xenopus laevis cDNA libraries containing Xenopus laevis BMP cDNA can be obtained by numerous techniques well known in the art including purchasing them from the market, though obtainable by the methods described above. For example, the cDNA library of Xenopus laevis is available from Clontech Laboratories, Inc., U.S.A.

Methods for cloning the Xenopus laevis BMP DNA from the Xenopus laevis DNA library include, for example, the plaque hybridization method using phage vector λcharon 28A and rat inhibin (activin) βA cDNA as probes [T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratory, (1982)].

The Xenopus laevis BMP DNA thus cloned is subcloned in plasmids such as pBR322, pUC12, pUC13, pUC19, pUC118 and pUC119 to obtain the Xenopus laevis BMP DNA, if necessary.

The nucleotide sequence of the DNA sequence thus obtained is determined, for example, by the Maxam-Gilbert method [A. M. Maxam and W. Gilbert, Proc. Natl. Acad. Sci. U.S.A. 74, 560 (1977)] or the dideoxy method [J. Messing et al., Nucleic Acids Research 9, 309 (1981)], and the existence of the Xenopus laevis BMP DNA is confirmed in comparison with the known amino acid sequence.

As described above, the DNA sequence [Xenopus laevis BMP DNAs represented by formulae (1) to (8)] coding for the Xenopus laevis BMPs are obtained.

Fig. 1 shows the restriction enzyme fragment maps of the DNA sequences containing the DNA segments coding for the Xenopus laevis BMPs obtained in Example 1 described below. Fig. 2 shows the nucleotide sequences represented by formulae (1) to (8) of the DNA sequences as determined by the dideoxy method, and Figs. 3 and 4 show the amino acid sequences represented by formulae (I) to (V) and formulae (VI) to (VIII), respectively, which were ascertained form the above nucleotide sequences.

The DNA sequence coding for the Xenopus laevis BMP cloned as described above can be used as it is, or after digestion with a restriction enzyme if desired, depending on the intended use.

A region intended to be expressed is cut out from the cloned DNA and ligated downstream from the promoter in

a vehicle (vector) suitable for expression, whereby the expression vector can be obtained.

The DNA sequence has ATG as a translation initiating codon at the 5'-terminus thereof and may have TAA, TGA or TAG as a translation terminating codon at the 3'-terminus. The translation initiating codon and translation terminating codon may be added by use of an appropriate synthetic DNA adaptor. The promoter is further ligated in the upstream thereof for the purpose of expressing the DNA sequence.

Examples of the vectors include the above plasmids derived from E. coli such as pBR322, pBR325, pUC12 and pUC13, the plasmide derived from B. subtilis such as pUB110, pTP5 and pC194, plasmids derived from yeast such as pSH19 and pSH15, bacteriophage such as λphage, and animal viruses such as retroviruses and vaccinia viruses.

As the promoters used in the present invention, any promoters are appropriate as long as they are suitable for expression in the host cells selected for the gene expression.

When the host cell used for transformation is Escherichia, it is preferable that a trp promoter, a lac promoter, a recA promoter, a λPL promoter, a lpp promoter, etc. are used. When the host cell is Bacillus, it is preferable that a PHO5 promoter, a PGK promoter, a GAP promoter, an ADH promoter, etc. are used. In particular, it is preferable that the host cell is Escherichia and the promoter is the trp promoter or the λPL promoter.

When the host cell is an animal cell, an SV-40 derived promoter, a retrovirus promoter, a metallothionein promoter, a heat shock promoter, etc. are each usable.

An enhancer, a certain DNA sequence important for promoter activity in a cell, is also effectively used for expression.

By using the vector containing the DNA sequence coding for the Xenopus laevis BMP mature peptide thus constructed, the transformant is prepared.

The host cell include, for example, Escherichia, Bacillus, yeast and animal cells.

Specific examples of the above Escherichia and Bacillus include strains similar to those described above.

Examples of the above yeast include Saccharomyces cerevisiae AH22, AH22R⁻, NA87-11A and DKD-5D.

Examples of animal cells include monkey cell COS-7, Vero, Chinese hamster cell (CHO), mouse L cell and human FL cell.

The transformation of the above Escherichia is carried out, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A. 69, 2110 (1972) or Gene 17, 107 (1982).

The transformation of the above Bacillus is conducted, for example, according to the method described in Molecular & General Genetics 168, 111 (1979).

The transformation of the yeast is carried out, for example, according to the method described in Proc. Natl. Acad. Sci. U.S.A. 75, 1929 (1978).

The transformation of the animal cells is carried out, for example, according to the method described in Virology 52, 456 (1973).

Thus, there is obtained the transformant transformed with the expression vector containing the DNA sequence coding for the Xenopus laevis BMP mature peptide.

When bacterial transformants are cultured, a liquid medium is particularly suitable as a medium used for culture. Carbon sources, nitrogen sources, inorganic compounds and others necessary for growth of the transformant are contained therein. Examples of the carbon sources include glucose, dextrin, soluble starch and sucrose. Examples of the nitrogen sources include inorganic or organic materials such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extracts, soybean meal and potato extract solution. The inorganic compounds include, for example, calcium chloride, sodium dihydrogenphosphate and magnesium chloride. Yeast extract, vitamins, growth promoting factors and so on may be further added thereto.

The pH of the medium is preferably about 5 to 8.

As the medium used for cultivation of Escherichia, there is preferred, for example, M9 medium containing glucose and Casamino Acids (Miller, Journal of Experiments in Molecular Genetics 431-433, Cold Spring Harbor Laboratory, New York, 1972). In order to make the promoter act efficiently, a drug such as 3β-indolylacrylic acid may be added thereto if necessary.

When the host cell is Escherichia, the cultivation is usually carried out at about 15 to 43°C for about 3 to 24 hours, with aeration or agitation if necessary.

When the host cell is Bacillus, the cultivation is usually carried out at about 30 to 40°C for about 6 to 24 hours, with aeration or agitation if necessary.

When yeast transformants are cultured, there is used, for example, Burkholder minimum medium [K. L. Bostian et al., Proc. Natl. Acad. Sci. U.S.A. 77, 4505 (1980)] as the medium. The pH of the medium is preferably adjusted to about 5 to 8. The cultivation is usually carried out at about 20 to 35°C for about 24 to 72 hours, with aeration or agitation if necessary.

When animal cell transformants are cultured, examples of the media include MEM medium containing about 5 to 20% fetal calf serum [Science 122, 501 (1952)], DMEM medium [Virology 8, 396 (1959)], RPMI1640 medium [Journal of the American Medical Association 199, 519 (1967)] and 199 medium [Proceeding of the Society for the Biological

Medicine 73, 1 (1950)]. The pH is preferably about 6 to 8. The cultivation is usually carried out at about 30 to 40°C for about 15 to 60 hours, with aeration or agitation if necessary.

The above Xenopus laevis BMP mature peptide can be isolated and purified from the culture described above, for example, by the following method.

When the Xenopus laevis BMP mature peptide is to be extracted from the cultured cells, the cells are collected by methods known in the art after cultivation. Then, the collected cells are suspended in an appropriate buffer solution and disrupted by ultrasonic treatment, lysozyme and /or freeze-thawing. Thereafter, a crude extracted solution of the Xenopus laevis BMP mature peptide is obtained by centrifugation or filtration. The buffer solution may contain a protein denaturant such as urea or guanidine hydrochloride, or a surface-active agent such as Triton X-100.

When the Xenopus laevis BMP precursor protein or mature peptide is secreted in the culture solution, a supernatant is separated from the cells by methods known in the art after the conclusion of cultivation, and then collected.

The separation and purification of the Xenopus laevis BMP precursor protein or mature peptide contained in the culture supernatant or the extracted solution thus obtained can be performed by an appropriate combination of known separating and purifying methods. The known separating and purifying methods include methods utilizing solubility such as salt precipitation and solvent precipitation, methods mainly utilizing a difference in molecular weight such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, methods utilizing a difference in electric charge such as ion-exchange column chromatography, methods utilizing specific affinity such as affinity chromatography, methods utilizing a difference in hydrophobicity such as reverse phase high performance liquid chromatography and methods utilizing a difference in isoelectric point such as isoelectro-focusing electrophoresis. Methods using an antibody to a fused protein expressed by fusing BMP complementary DNA or DNA with E. coli-derived DNA lacZ can also be used.

Illustrative examples of the methods for expressing the BMP in the present invention include methods in which genes are introduced into CHO cells to produce the BMP in large amounts as described in Wang et al., Proc. Natl. Acad. Sci. U.S.A. 807, 2220-2224 (1990).

The activity of the Xenopus laevis BMP precursor protein or mature peptide thus formed can be measured by an enzyme immunoassay using a specific antibody. If the products have a bone morphogenetic activity, this activity may also be measured as an index.

The cells, such as animal cells or E. coli, transinfected or transformed with the DNA sequences of the present invention allow large amounts of the Xenopus laevis BMP mature peptides to be produced. Hence, the production of these peptides can be advantageously achieved.

It has become clear that the Xenopus laevis BMP mature peptides prepared here promote the synthesis of proteoglycan which is a main component of a cartilage matrix, and the peptides can also be utilized for analysis of the mechanism of organism, particularly human bone-cartilage morphogenetic reaction, and as therapeutic agents for fracture or osteoporosis.

In such instances one would administer an effective amount of the protein to a mammal. An effective amount is the amount of protein needed to promote the synthesis of proteoglycan in cartilage cells. Typically, this ranges from 0.001 to 35 µg per kg/body weight. The precise amount for a particular purpose can readily be determined empirically by the person of ordinayl skill in the art based upon the present disclosure.

When one uses the protein for therapeutic purpose care is taken to purify it and render it substantially free of bacterica and pyrogens. This can be done by standard methods.

When the BMPs are used as therapeutic agents for fracture or osteoporosis, they can be administered parenterally in the forms of solutions, injections and ointments, solely or in combination with pharmaceutically acceptable additional components, such as vehicles, binders, dispersants, plasticizers or diluents.

The preferable administration forms include (1) administration of the agent to cutis surface near a diseased part, (2) injection of the agent into a diseased part, (3) discission of a diseased part followed by direct administration of the agent thereto. The preferable dose in fractue therapy for adult people is 0.1 to 2000 µg more, preferably 20 to 400 µg for adult people once a day. The preferable dose in osteoporosis for adult people is 0.1 to 200 µg once a day, for about one to 30 days. The concentration of the therapeutic agent is, preferably, 0.001 to 0.2% in the form of a solution, 0.001 to 0.2% in the form of an injections, and 0.0001 to 0.2% in the form of an ointment.

There have been described above in detail the cloning of the DNA sequences coding for the Xenopus laevis BMPs, the preparation of the expression vectors for the Xenopus laevis BMP mature peptides, the production of the transformants by using the transformants and their utility.

When nucleotides, amino acids and so on are indicated by the abbreviations in this specification and drawings, the abbreviations adopted by IUPAC-IUB Commission on Biochemical Nomenclature or commonly used in the art are employed. For example, the following abbreviations are used. When the amino acids are capable of existing as optical isomer, the L-forms are represented unless otherwise specified.

DNA : Deoxyribonucleic acid

cDNA :	Complementary deoxyribonucleic acid
A :	Adenine
T :	Thymine
G :	Guanine
C :	Cytosine
RNA :	Ribonucleic acid
mRNA :	Messenger ribonucleic acid
dATP :	Deoxyadenosine triphosphate
dTTP :	Deoxythymidine triphosphate
dGTP :	Deoxyguanosine triphosphate
dCTP :	Deoxycytidine triphosphate
ATP :	Adenosine triphosphate
EDTA :	Ethylenediaminetetraacetic acid
SDS :	Sodium dodecyl sulfate
Gly or G :	Glycine
Ala or A :	Alanine
Val or V :	Valine
Leu or L :	Leucine
Ile or I :	Isoleucine
Ser or S :	Serine
Thr or T :	Threonine
Cys or C :	Cysteine
Met or M :	Methionine
Glu or E :	Glutamic acid
Asp or D :	Aspartic acid
Lys or K :	Lysine
Arg or R :	Arginine
His or H :	Histidine
Phe or F :	Phenylalanine
Tyr or Y :	Tyrosine
Trp or W :	Tryptophan
Pro of P :	Proline
Asn or N :	Asparagine
Gln or Q :	Glutamine

With respect to the Xenopus laevis BMP mature peptides of the present invention, a portion of the amino acid sequence may be modified, namely there may be addition, elimination or substitution with other amino acids as long as the bone morphogenetic activity is not lost.

The present invention will hereinafter be described in detail with the following Examples. It is understood of course that these Examples are not intended to limit the scope of the invention.

Transformants E. coli HB101/pXar3 (coding for protein M3), E. coli HB101/pXar4 (coding for protein A4), E. coli HB101/pXar5 (coding for protein A5), E. coliHB101/pXar9 (coding for protein B9) and E. coli HB101/pXar14 (coding for protein C4) each obtained in Example 1 described below were deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession numbers IFO 14928, IFO 14929, IFO 14930, IFO 14931 and IFO 14932, respectively, on August 28, 1989. These transformants were also deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the Budapest Treaty under the accession numbers FERM BP-2578, FERM BP-2579, FERM BP-2580, FERM BP-2581 and FERM BP-2582, respectively, on September 2, 1989.

The transformants E. coli HB101/pXbr22 (coding for Xenopus laevis BMP-2A), E. coli HB101/pXbr23 (coding for Xenopus laevis BMP-2B) and E. coli HB101/pXbr41 (coding for protein Xenopus laevis Vgr-1) each obtained in Example 2 described below were deposited with the Institute for Fermentation, Osaka, Japan (IFO) under the accession numbers IFO 15080, IFO 15081 and IFO 15082, respectively, on August 10, 1990. These transformants were also deposited with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan (FRI) under the Budapest Treaty under the accession numbers FERM BP-3066, FERM BP-3065 and FERM BP-3067, respectively, on August 16, 1990.

Example 1

Preparation of Xenopus laevis Liver-Derived DNA Library

(1) Preparation of Xenopus laevis Chromosome DNA

The liver (1 g) of Xenopus laevis was powdered in liquid nitrogen, and 10 ml of buffer (1) [100 μg/ml proteinase K, 0.5% Sarkosil, 0.5 M EDTA (pH 8.0)] was added thereto, followed by incubation at 50°C for 2 hours. The resulting DNA sample was treated with phenol, and then dialyzed against buffer (2) [10 mM EDTA, 10 mM NaCl, 50 mM Tris-HCl (pH 8.0)] to remove phenol. RNase was added thereto to a final concentration of 100 μg/ml, and the mixture was incubated at 37°C for 3 hours, followed by phenol treatment twice. The aqueous layer was dialyzed against buffer (3) [1 mM EDTA, 10 mM Tris-HCl (pH 8.0)]. Thus, about 1 mg of liver-derived chromosome DNA was obtained. This DNA (10 μg) was partially cleaved with restriction enzyme Sau3AI, and the resulting product was subjected to equilibrium density gradient centrifugation using CsCl. Fractions containing DNA fragments having lengths of 10 to 20 kb were selected and introduced into fragments obtained by cleaving phage charon 28 DNA with BamHI and used as a vector. This reaction called "ligation" was conducted at 15°C for 16 hours. The charon 28 vector into which the Xenopus laevis chromosome DNA was thus introduced was contained in a phage head (in vitro packaging). This procedure was carried out by using a commercial packaging kit (Gigapack Gold, Stratagene). This recombinant phage was amplified by infection with E. coli LE392. Specifically, the phage was mixed with excess LE392 to allow LE392 to adsorb the phage at 37°C for 10 minutes. Then, the mixture was plated on NZYM medium (containing 13% agar), followed by incubation overnight.

(2) Screening

The total number of the phage clones was estimated to be about 1,000,000 from the number of the plaques produced in a dish. As a probe (DNA used for detection of a desired gene by hybridization), there was used rat activin βA cDNA [Molecular Endocrinology 1, 388-396 (1987)] labeled with ^{32}P by a random priming method. The plaques transcribed from the dish to a nitrocellulose membrane were returned to neutrality (0.2 M Tris, 0.6 M NaCl, pH 7.4) through alkali treatment (immersion in 0.1 N NaOH, 0.6 M NaCl for 30 seconds). After completion of the treatment described above, the membrane was heated in a vacuum thermostat at 80°C for 1 hour. After heating, the membrane was immersed in a hybridization solution (50% formamide, 5 X Denhardt's solution, 5 X SSPE, 0.1% SDS, 100 μg/ml denatured salmon sperum DNA) to incubate it at 42°C for 4 hours. Then, the membrane was allowed to stand in the mixture solution of the above hybridization solution and the DNA probe at 60°C overnight. This procedure was carried out in a plastic bag. The next day, the nitrocellulose membrane was taken out of the bag, and washed with a solution of 2 X SSC and 0.1% SDS for 15 minutes and with a solution of 0.1 X SSC and 0.1% SDS for 15 minutes, increasing the temperature stepwise, until the cpm value of the membrane reached about 1,000 cpm. After washing, the washing solution was removed by filter paper, and then the membrane was subjected to autoradiography. The plaque containing the desired gene was identified by exposure of a Fuji X-ray film. The genes were cloned by repetition of the above plaque hybridization.

20 X SSC contains 0.3 M sodium citrate (pH 7.0) and 3 M NaCl; 20 X SSPE contains 0.2 M sodium phosphate, 20 m EDTA and 3 M NaCl (pH 7.4); and Denhardt's solution contains 1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA (Pentex Fraction V).

(3) Determination of Nucleotide Sequence (Sequencing)

All of the five isolated clones A4, A5, B9, C4 and M3 were each subcloned into plasmid pUC19. In subcloning each clone into plasmid pUC19, subcloning was carried out utilizing a restriction enzyme recognition site which produced a fragment hybridized with the probe for each clone. However, for cloning clone A4, a commercial Bg1II linker was used to ligate a SmaI site.

The plasmids were each transformed into competent cell HB101 (E. coli) prepared by the rubidium chloride method to obtain five kinds of transformants E. coli HB101/pXar3 (coding for protein M3), E. coli HB101/pxar4 (coding for protein A4), E. coli HB101/pxar5 (coding for protein A5), E. coli HB101/pXar9 (coding for protein B9) and E. coli HB101/pXar14 (coding for protein C4), respectively.

For determination of the nucleotide sequence, a deletion mutant of each clone was prepared, and the shortest of fragment hybridized with the probe was selected. The nucleotide sequence was determined from pUC19 by the direct Sanger method (or the dideoxy method).

For translation of the nucleotide sequence to an amino acid sequence or for screening of homology, a software for genetic analysis (GENETYX, Nippon SDC) was used.

Homology at Nucleic Acid Level						
TYX nucleotide	Rat Act βA, %	Rat Act βA, %	Human TGF β2, %	xVgl %	M3 %	A4 %
A5	70.3 (101)	47.5 (314)	43.8 (169)	48.5 (171)	54.7 (258)	63.7 (328)
A4	69.5 (0.5)	-	-	-	55.4 (251)	
M3	63.6 (332)	53.9 (672)	33.1 (689)	-		

In the above table, numerical values in parentheses indicate the length compared (bp).

Homology at Amino Acid Level						
TYX nucleotide	Rat Act βA, %	Rat Act βA, %	Human TGF β2, %	xVgl %	M3 %	A4 %
A5	58.8 (34)	44.1 (34)	37.2 (43)	50.0 (38)	26.0 (77)	67.6 (68)
A4	41.3 (63)	44.1 (34)	39.5 (43)	52.6 (38)	30.3 (66)	
M3	50.3 (149)	49.4 (162)	32.8 (128)	40.6 (106)		

In the above table, numerical values in parentheses indicate the length compared (bp).

Example 2

Preparation of Xenopus laevis Unfertilized Egg-Derived DNA Library

(1) Preparation of Xenopus laevis BMP-2A Probe

A probe was prepared by fragmentation of chromosome DNA Xarl4 coding for Xenopus laevis BMP-2A with restriction enzymes PstI and HindIII, and three kinds of cDNAs, Xbr22, Xbr23 and Xbr41 were isolated by screening of a Xenopus laevis unfertilized egg cDNA library by a hybridization method. The comparison with the structure of the Xenopus laevis BMP chromosome DNA already isolated revealed that Xbr22, Xbr23 and Xbr41 coded for proteins having homology with Xenopus laevis BMP-2A, Xenopus laevis BMP-2B and mouse Vgr-1 reported by Lyon et al. [Proc. Natl. Acad. Sci. U.S.A. 806, 4554-4558 (1989)], respectively.

The Xenopus laevis unfertilized egg cDNA library was provided by the Salk Institute (C. Kintner). This library was prepared based on λgt10. This recombinant phage was amplified by infection with E. coli NM514. Specifically, the phage was mixed with excess NM514 to allow NM514 to adsorb the phage at 37°C for 10 minutes. Then, the mixture was plated on NZYM medium (containing 13% agar), followed by incubation overnight.

(2) Screening

The total number of the phage clones was estimated to be about 1,200,000 from the number of the plaques produced in a dish. As a probe (DNA used for detection of a desired gene by hybridization), there was used a DNA fragment (185 bp) obtained by cleaving Xar14 with restriction enzymes PstI and HindIII and labeled with ^{32}P by a random priming method. The plaques transcribed from the dish to a nitrocellulose membrane were returned to neutrality (0.2 M Tris, 0.6 M NaCl, pH 7.4) through alkali treatment (immersion in 0.1 N NaOH, 0.6 M NaCl for 30 seconds). After completion of the treatment described above, the membrane was heated in a vacuum thermostat at 80°C for 1 hour. After heating, the membrane was immersed in a hybridization solution (50% formamide, 5 X Denhardt's solution, 5 X SSPE, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA) to incubate it at 42°C for 4 hours. Then, the membrane was allowed to stand in the mixture solution of the above hybridization solution and the DNA probe at 60°C overnight. This procedure was carried out in a plastic bag. The next day, the nitrocellulose membrane was taken out of the bag, and washed with a solution of 2 X SSC and 0.1% SDS for 15 minutes, increasing the temperature stepwise, until the cpm value of the membrane reached about 1,000 cpm. After washing, the washing solution was removed by filter paper, and then the membrane was subjected to autoradiography. The plaque containing the desired gene was identified by exposure of a Fuji X-ray film. The genes were cloned by repetition of the above plaque hybridization.

20 X SSC contains 0.3 M sodium citrate (pH 7.0) and 3 M NaCl; 20 X SSPE contains 0.2 M sodium phosphate, 20 m EDTA and 3 M NaCl (pH 7.4); and Denhardt's solution contains 1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA (Pentex Fraction V).

(3) Determination of Nucleotide Sequence (Sequencing)

All of the three isolated clones Xbr22, Xbr23 and Xbr41 were each subcloned into plasmid pUC19. In subcloning each clone into plasmid pUC19, subcloning was carried out utilizing a restriction enzyme recognition site which produced a fragment hybridized with the probe for each clone.

The plasmids were each transformed into competent cell HB101 (E. coli) prepared by the rubidium chloride method to obtain three kinds of transformants E. coli HB101/pXbr22 (coding for Xenopus laevis BMP-2A), E. coli HB101/pXbr23 (coding for Xenopus laevis BMP-2B) and E. coli HB101/pXbr41 (coding for protein Xenopus laevis Vgr-1), respectively.

For determination of the nucleotide sequence, a deletion mutant of each clone was prepared, and the shortest fragment that hybridized with the probe was selected. The nucleotide sequence was determined from pUC19 by the direct Sanger method (or the dideoxy method).

For translation of the nucleotide sequence to an amino acid sequence or for screening of homology, a software for genetic analysis (GENETYX, Nippon SDC) was used.

Figs. 2(6) to 2(8) show the respective nucleotide sequences, and Figs. 4(VI) to 4(VIII) show the respective amino acid sequences.

Example 3

In order to examine the biological activity of the Xenopus laevis BMP-related gene products, each of Xbr22, Xbr23 and Xbr41 cDNAs was inserted into expression vector pCDM8 (Invitrogen, U.S.A.) for animal cells, and expressed in a COS cell(African green monkey kidney cell). The resulting culture supernatant was used for determination of the biological activity.

Each of the Xbr22, Xbr23 and Xbr41 cDNAs to which XhoI linkers were ligated at both ends thereof was inserted into the XhoI restriction enzyme-cleaving site of pCDM8 to use it for transfection (introduction of DNA). 3 X 10^6 cells were subcultured in a 100 mm diameter plastic dish, and the medium was removed after 24 hours, followed by washing once with 10 ml of TBS (Tris-buffered saline). 300 µl of a DNA solution (1.5 µg DNA) diluted with TBS was mixed with 300 µl of a 0.1% DEAE-dextran solution, and the combined solution was added dropwise to the cells. After standing at ordinary temperature for 15 minutes, the cells were washed once with 300 µl of TBS, and then incubated in Dulbecco's modified Eagle's medium (DMEM, containing 10% FBS, 100 U/ml penicillin, 100 mcg/ml streptomycin and 100 uM chloroquine). After 3 hours, the cells were washed twice with TBS and incubated in DMEM (containing 10% FBS, 100 U/ml penicillin and 100 mcg/ml streptomycin). After 24 hours, the cells were washed three times with TBS and incubated in DMEM (containing 100 U/ml penicillin and 100 mcg/ml streptomycin) for 4 days, followed by recovery of the medium. The recovered medium was centrifuged at 2,000 rpm for 5 minutes to obtain a culture supernatant.

The culture supernatant thus obtained was used for determination of the biological activity as a sample containing Xenopus laevis BMP2-A, BMP-2B or protein Vgr-1. Namely, each of the samples was added to the medium of rabbit chondrocytes in monolayer cultures [Y. Kato et al., Exp. Cell Res. 130, 73-81 (1980); Y. Kato et al., J. Biol. Chem. 265, 5903-5909 (1990)] to examine their effect on the synthesis of proteoglycan, the main component of a cartilage matrix. As a result, the control in which the COS cell was transfected with the expression vector alone and the medium conditioned by untreated COS cells did not affect the synthesis of proteoglycan, as shown in the following table. In contrast, the above three kinds of proteins obtained in the present invention strongly promoted the synthesis of proteoglycan by the cartilage cells. The maximum activity of Xenopus laevis BMP-2A, BMP- 2B and Vgr-1 was stronger than that of TGF-beta-1. The synthesis of proteoglycan was determined by measuring ^{35}S-sulfate incorporation into glycosaminoglycans [Y. Kato et al., Exp. Cell Res. 130, 73-81 (1980); Y. Kato et al., J. Biol. Chem. 265, 5903-5909 (1990)]. These results show that the BMPs of Xenopus laevis promote the differentiation of cartilages, and suggest that the BMPs of other animals have similar effects. The BMPs are therefore expected to be applied to therapeutic agents for healing acceleration of fractures and for various diseases of cartilages and bones (such as arthritis and osteoporosis).

* Kind of Cell

Rabbit costal chondrocytes maintained on 6-mm diameter plastic wells.

* Kind of Marker

^{35}S µCi/ in 100 µl medium per well

* Kind of Medium

A 1:1 (V/V) mixture of DMEM and Ham's F-12 medium supplemented with 0.3% fetal bovine serum.

No.	Additive	Count			Mean ± S.D.	% to Control
1	Control	5193	4328	4269	4695 ± 351	100
		4565	4727	5089		
2	xBMP2A 1 5µl	2362	2749	2758	2362 ± 185	56
3	xBMP2A 1/3 5µl	12198	15502	21891	16530 ± 4023	352
4	xBMP2A 1/10 5µl	10004	9738	8848	9530 ± 494	203
5	xBMP2B 1 5µl	3171	2906	3219	3099 ± 138	66
6	xBMP2B 1/3 5µl	11315	9750	13139	11401 ± 1385	243
7	xBMP2B 1/10 5µl	12426	13457	13324	13069 ± 458	278
8	xVgr-1 1 5µl	5188	2833	4416	4146 ± 980	88
9	xVgr-1 1/3 5µl	7486	8834	7202	7841 ± 712	167
10	xVgr-1 10 5µl	15286	15645	13032	14654 ± 1156	312
11	pCDM8 5µl	3604	2694	2927	3075 ± 386	65
12	pCMD8 1µl	2637	4219		3428 ± 791	73
13	DNA(-) 5µl	3625	4050	4714	4130 ± 448	88
14	DNA(-) 1µl	5695	4657		5176 ± 519	110
15	DME 5µl	3614	8963	3850	5476 ± 2468	117
16	DME 1µl	4384	3874	5760	4675 ± 799	100
17	TGF-B1 3ng/ml	9381	12474	10922		
		10058	11546	11155	10923 ± 998	233
18	Ins. 5 g/ml	19431	20476	22746		
		25066	27835	24965	23420 ± 2876	499
19	Ins. 3 g/ml	13620	15378	11987		
		11240	12699	12666	12932 ± 1313	275

pCDM8: A culture solution of the cells into which pCDM8 is introduced as a vector

DNA(-): A culture solution which is in contact with the cells, which do not produce the BMPs

DME: A solution which is not in contact with the cells

Ins.: Insulin

Experiments Procedure

Rabbit chondrocytes were isolated from growth plates of ribs of 3- to 4- week old male New Zealand rabbits, as previously described (Y. Kato et al. Exp. Cell Res.). Cells were seeded at a density 10^4 cells / 6-mm diameter plastic culture well in 0.1 ml of Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum and antibiotics. When cultures became confluent, the cells were preincubated for 24 hours in 0.1 ml of a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 0.3% fetal bovine serum (DF). The cells were then transferred to 0.1 ml of the same medium (DF) supplemented with 1 or 5 µl of the medium that was conditioned by various COS cells: [The conditioned medium was diluted or not diluted with DMEM (a final concentration of 10 or 30%)]. After 3 hours, 5 µl of DMEM supplemented with 1µCi of $^{35}SO_4^{2-}$ was also added, and incubation was continued for a further 17 hours (Y. Kato et al. Exp. Cell Res.).

Claims

Claims for the following Contracting States : DE, GB, FR, IT, NL, SE, CH, LI, BE, AT, LU, GR, DK

1. A Xenopus laevis bone morphogenetic protein, wherein said protein is a mature protein containing an amino acid sequence having an amino acid sequence represented by Nos. 15 to 130 of formula (I) shown in Fig. 3, an amino acid sequence represented by Nos. 14 to 127 of formula (II) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 63 of formula (IV) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 65 of formula (V) shown in Fig. 3, an amino acid sequence represented by Nos. 282 to 398 or Nos. 298 to 398 of formula (VI) shown in Fig. 4, or an amino acid sequence represented by Nos. 328 to 426 of formula (VIII) shown in Fig. 4.

2. A Xenopus laevis bone morphogenetic protein, wherein said protein is a precursor protein containing an amino acid sequence having an amino acid sequence represented by formula (I), (II), (IV) or (V) shown in Fig. 3, or formula (VI) or (VIII) shown in Fig. 4.

3. A DNA comprising a DNA segment coding for a Xenopus laevis bone morphogenetic protein according to claim 1 or 2.

4. A DNA comprising a DNA segment coding for a Xenopus laevis bone morphogenetic protein, wherein said DNA segment comprises a nucleotide sequence corresponding to the nucleotide sequence represented by formula (1), (2), (3), (4), (5), (6), (7) or (8) shown in Fig. 2.

5. A non-human transformant bearing a DNA comprising a DNA segment according to claim 3 or 4, wherein said transformant is not Xenopus laevis.

6. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXar3 (FERM BP-2578).

7. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXar4 (FERM BP-2579).

8. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXar5 (FERM BP-2580).

9. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXar9 (FERM BP-2581).

10. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXar14 (FERM BP-2582).

11. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXbr22 (FERM BP-3066).

12. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXbr23 (FERM

BP-3065).

13. A transformant in accordance with claim 5, which has the characteristics of Escherichia coli HB101/pXbr41 (FERM BP-3067).

14. A method for preparing a Xenopus laevis bone morphogenetic protein according to claim 1 or 2 which method comprises culturing a non-human transformant as defined in claim 5, producing and accumulating the protein in a culture, and collecting the protein thus obtained.

15. A composition for therapy of fracture or osteoporosis which contains an effective amount of a Xenopus laevis bone morphogenetic protein according to claim 1 or 2 and pharmaceutically acceptable additional components.

16. A method for preparing a composition for therapy of fracture or osteoporosis which comprises admixing an effective amount of a Xenopus laevis bone morphogenetic protein according to claim 1 or 2 with pharmaceutically acceptable additional components.

Claims for the following Contracting State : ES

1. A method for preparing a Xenopus laevis bone morphogenetic protein, wherein said protein is a mature protein containing an amino acid sequence having an amino acid sequence represented by Nos. 15 to 130 of formula (I) shown in Fig. 3, an amino acid sequence represented by Nos. 14 to 127 of formula (II) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 63 of formula (IV) shown in Fig. 3, an amino acid sequence represented by Nos. 6 to 65 of formula (V) shown in Fig. 3, an amino acid sequence represented by Nos. 282 to 398 or Nos. 298 to 398 of formula (VI) shown in Fig. 4, or an amino acid sequence represented by Nos. 328 to 426 of formula (VIII) shown in Fig. 4, or wherein said protein is a precursor protein containing an amino acid sequence having an amino acid sequence represented by formula (I), (II), (IV) or (V) shown in Fig. 3, or formula (VI) or (VIII) shown in Fig. 4, which method comprises culturing a non-human transformant bearing a DNA comprising a DNA segment coding for the protein, wherein said transformant is not Xenopus laevis, producing and accumulating the protein in a culture, and collecting the protein thus obtained.

2. A method for preparing a DNA comprising a DNA segment coding for a Xenopus laevis bone morphogenetic protein as defined in claim 1, which method comprises cloning the DNA from a Xenopus laevis DNA library.

3. A method for preparing a DNA comprising a DNA segment coding for a Xenopus laevis bone morphogenetic protein, wherein said DNA segment comprises a nucleotide sequence corresponding to the nucleotide sequence represented by formula (1), (2), (3), (4), (5), (6), (7), or (8) shown in Fig. 2, which method comprises cloning the DNA from a Xenopus laevis DNA library.

4. A method for preparing a non-human transformant bearing a DNA comprising a DNA segment as prepared in claim 3 or 4, wherein the prepared transformant is not Xenopus laevis, which method comprises transforming a host with said DNA.

5. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXar3 (FERM BP-2578).

6. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXar4 (FERM BP-2579).

7. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXar5 (FERM BP-2580).

8. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXar9 (FERM BP-2581).

9. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXar14 (FERM BP-2582).

10. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXbr22 (FERM BP-3066).

11. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXbr23 (FERM BP-3065).

12. A method in accordance with claim 4, wherein the prepared transformant has the characteristics of Escherichia coli HB101/pXbr41 (FERM BP-3067).

13. A method for preparing a composition for therapy of fracture or osteoporosis which method comprises admixing an effective amount of a Xenopus laevis bone morphogenetic protein as prepared according to claim 1 with pharmaceutically acceptable additional components.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : DE, GB, FR, IT, NL, SE, CH, LI, BE, AT, LU, GR, DK

1. *Xenopus laevis*-Knochenmorphogenese-Protein, wobei das Protein ein reifes Protein ist, das eine Aminosäurensequenz mit einer Aminosäurensequenz, welche durch die Nr. 15 bis 130 der in Fig. 3 dargestellten Formel (I) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 14 bis 127 der in Fig. 3 dargestellten Formel (II) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 6 bis 63 der in Fig. 3 dargestellten Formel (IV) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 6 bis 65 der in Fig. 3 dargestellten Formel (V) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 282 bis 398 oder Nr. 298 bis 398 der in Fig. 4 dargestellten Formel (VI) dargestellt wird, oder einer Aminosäurensequenz, welche durch die Nr. 328 bis 426 der in Fig. 4 dargestellten Formel (VIII) dargestellt wird, enthält.

2. *Xenopus laevis*-Knochenmorphogenese-Protein, wobei das Protein ein Vorläuferprotein ist, das eine Aminosäurensequenz mit einer Aminosäurensequenz enthält, welche durch die in Fig. 3 dargestellte Formel (I), (II), (IV) oder (V) oder in Fig. 4 dargestellte Formel (VI) oder (VIII) dargestellt wird.

3. DNA umfassend ein DNA-Segment, das für ein *Xenopus laevis*-Knochenmorphogenese-Protein gemäß Anspruch 1 oder 2 kodiert.

4. DNA umfassend ein DNA-Segment, das für ein *Xenopus laevis*-Knochenmorphogenese-Protein kodiert, wobei das DNA-Segment eine Nukleotidsequenz umfaßt, die der Nukleotidsequenz entspricht, welche durch die in Fig. 2 dargestellte Formel (1), (2), (3), (4), (5), (6), (7) oder (8) dargestellt wird.

5. Nicht-humane Transformante, die eine DNA trägt, welche ein DNA-Segment gemäß Anspruch 3 oder 4 umfaßt, wobei die Transformante nicht *Xenopus laevis* ist.

6. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXar3 (FERM BP-2578) hat.

7. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXar4 (FERM BP-2579) hat.

8. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXar5 (FERM BP-2580) hat.

9. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXar9 (FERM BP-2581) hat.

10. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXar14 (FERM BP-2582) hat.

11. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXbr22 (FERM BP-

3066) hat.

12. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXbr23 (FERM BP-3065) hat.

13. Transformante gemäß Anspruch 5, welche die Eigenschaften von *Escherichia coli* HB101/pXbr41 (FERM BP-3067) hat.

14. Verfahren zum Herstellen eines *Xenopus laevis*-Knochenmorphogenese-Proteins gemäß Anspruch 1 oder 2, wobei das Verfahren das Kultivieren einer in Anspruch 5 definierten nicht-humanen Transformanten, das Erzeugen und Anreichern des Proteins in einer Kultur und Isolieren des auf diese Weise erhaltenen Proteins umfaßt.

15. Zusammensetzung zur Fraktur- oder Osteoporosetherapie, die eine wirksame Menge eines *Xenopus laevis*-Knochenmorphogenese-Proteins gemäß Anspruch 1 oder 2 und pharmazeutisch annehmbare Zusatzbestandteile enthält.

16. Verfahren zum Herstellen einer Zusammensetzung zur Fraktur- oder Osteoporosetherapie, welches das Mischen einer wirksamen Menge eines *Xenopus laevis*-Knochenmorphogenese-Proteins gemäß Anspruch 1 oder 2 mit pharmazeutisch annehmbaren Zusatzbestandteilen umfaßt.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zum Herstellen eines *Xenopuslaevis*-Knochenmorphogenese-Proteins, wobei das Protein ein reifes Protein ist, das eine Aminosäurensequenz mit einer Aminosäurensequenz, welche durch die Nr. 15 bis 130 der in Fig. 3 dargestellten Formel (I) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 14 bis 127 der in Fig. 3 dargestellten Formel (II) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 6 bis 63 der in Fig. 3 dargestellten Formel (IV) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 6 bis 65 der in Fig. 3 dargestellten Formel (V) dargestellt wird, einer Aminosäurensequenz, welche durch die Nr. 282 bis 398 oder Nr. 298 bis 398 der in Fig. 4 dargestellten Formel (VI) dargestellt wird, oder einer Aminosäurensequenz, welche durch die Nr. 328 bis 426 der in Fig. 4 dargestellten Formel (VIII) dargestellt wird, enthält oder wobei das Protein ein Vorläuferprotein ist, das eine Aminosäurensequenz mit einer Aminosäurensequenz enthält, welche durch die in Fig. 3 dargestellte Formel (I), (II), (IV) oder (V) oder in Fig. 4 dargestellte Formel (VI) oder (VIII) dargestellt wird, wobei das Verfahren das Kultivieren einer nicht-humanen Transformanten, die eine DNA trägt, welche ein DNA-Segment umfaßt, das für das Protein kodiert, wobei die Transformante nicht *Xenopus laevis* ist, das Erzeugen und Anreichern des Proteins in einer Kultur und Isolieren des auf diese Weise erhaltenen Proteins umfaßt.

2. Verfahren zum Herstellen einer DNA, die ein DNA-Segment umfaßt, das für das in Anspruch 1 definierte *Xenopus laevis*-Knochenmorphogenese-Protein kodiert, wobei das Verfahren das Klonieren der DNA aus einer *Xenopus laevis*-DNA-Bank umfaßt.

3. Verfahren zum Herstellen einer DNA, die ein DNA-Segment umfaßt, das für ein *Xenopus laevis*-Knochenmorphogenese-Protein kodiert, wobei das DNA-Segment eine Nukleotidsequenz umfaßt, die der Nukleotidsequenz entspricht, welche durch die in Fig. 2 dargestellte Formel (1), (2), (3), (4), (5), (6), (7) oder (8) dargestellt wird, wobei das Verfahren das Klonieren der DNA aus einer *Xenopus laevis*-DNA-Bank umfaßt.

4. Verfahren zum Herstellen einer nicht-humanen Transformante, die eine DNA trägt, welche ein in Anspruch 3 oder 4 hergestelltes DNA-Segment umfaßt, wobei die hergestellte Transfotmante nicht *Xenopus laevis* ist und das Verfahren das Transformieren eines Wirts mit der DNA umfaßt.

5. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXar3 (FERM BP-2578) hat.

6. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXar4 (FERM BP-2579) hat.

7. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli*

HB101/pXar5 (FERM BP-2580) hat.

8. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXar9 (FERM BP-2581) hat.

9. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXar14 (FERM BP-2582) hat.

10. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXbr22 (FERM BP-3066) hat.

11. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXbr23 (FERM BP-3065) hat.

12. Verfahren gemäß Anspruch 4, wobei die hergestellte Transformante die Eigenschaften von *Escherichia coli* HB101/pXbr41 (FERM BP-3067) hat.

13. Verfahren zum Herstellen einer Zusammensetzung zur Fraktur- oder Osteoporosetherapie, welches das Mischen einer wirksamen Menge eines gemäß Anspruch 1 hergestellten *Xenopus laevis*-Knochenmorphogenese-Proteins mit pharmazeutisch annehmbaren Zusatzbestandteilen umfaßt.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, GB, GR, IT, LI, LU, NL, SE

1. Protéine intervenant dans la morphogenèse des os de Xenopus laevis, qui est une protéine mature dont la séquence d'acides aminés comporte la séquence des acides aminés n° 15 à 130 de la formule (I) indiquée dans la figure 3, la séquence des acides aminés n° 14 à 127 de la formule (II) indiquée dans la figure 3, la séquence des acides aminés n° 6 à 63 de la formule (IV) indiquée dans la figure 3, la séquence des acides aminés n° 6 à 65 de la formule (V) indiquée dans la figure 3, la séquence des acides aminés n° 282 à 398 ou n° 298 à 398 de la formule (VI) indiquée dans la figure 4, ou la séquence des acides aminés n° 328 à 426 de la formule (VIII) indiquée dans la figure 4.

2. Protéine intervenant dans la morphogenèse des os de Xenopus laevis, qui est une protéine précurseur dont la séquence d'acides aminés comporte une séquence d'acides aminés représentée par l'une des formules (I), (II), (IV) et (V) indiquées dans la figure 3 ou par l'une des formules (VI) et (VIII) indiquées dans la figure 4.

3. ADN comportant un segment d'ADN codant une protéine intervenant dans la morphogenèse des os de Xenopus laevis, conforme à l'une des revendications 1 et 2.

4. ADN comportant un segment d'ADN codant une protéine intervenant dans la morphogenèse des os de Xenopus laevis, dans lequel ledit segment d'ADN comporte une séquence de nucléotides qui correspond à une séquence de nucléotides représentée par l'une des formules (1), (2), (3), (4), (5), (6), (7) et (8) indiquées dans la figure 2.

5. Individu transformé non-humain qui héberge un ADN comportant un segment d'ADN, conforme à l'une des revendications 3 et 4, mais qui n'est pas un individu Xenopus laevis.

6. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXar3 (FERM BP-2578).

7. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXar4 (FERM BP-2579).

8. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXar5 (FERM BP-2580).

9. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXar9 (FERM BP-2581).

10. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXar14 (FERM BP-2582).

11. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXbr22 (FERM BP-3066).

12. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXbr23 (FERM BP-3065).

13. Individu transformé conforme à la revendication 5, présentant les caractéristiques d'Escherichia coli HB101/pXbr41 (FERM BP-3067).

14. Procédé de préparation d'une protéine intervenant dans la morphogenèse des os de Xenopus laevis, conforme à l'une des revendications 1 et 2, lequel procédé comporte le fait de cultiver un individu transformé non-humain conforme à la revendication 5, le fait de laisser la protéine produite s'accumuler dans la culture, et le fait de recueillir la protéine ainsi obtenue.

15. Composition destinée au traitement des fractures ou de l'ostéoporose, qui contient une quantité efficace d'une protéine intervenant dans la morphogenèse des os de Xenopus laevis, conforme à l'une des revendications 1 et 2, et des composants supplémentaires admissibles en pharmacie.

16. Procédé de préparation d'une composition destinée au traitement des fractures ou de l'ostéoporose, qui comporte le fait de mélanger une quantité efficace d'une protéine intervenant dans la morphogenèse des os de Xenopus laevis, conforme à l'une des revendications 1 et 2, avec des composants supplémentaires admissibles en pharmacie.

Revendications pour l'Etat contractant suivant : ES

1. Procédé de préparation d'une protéine intervenant dans la morphogenèse des os de Xenopus laevis, qui est une protéine mature dont la séquence d'acides aminés comporte la séquence des acides aminés n° 15 à 130 de la formule (I) indiquée dans la figure 3, la séquence des acides aminés n° 14 à 127 de la formule (II) indiquée dans la figure 3, la séquence des acides aminés n° 6 à 63 de la formule (IV) indiquée dans la figure 3, la séquence des acides aminés n° 6 à 65 de la formule (V) indiquée dans la figure 3, la séquence des acides aminés n° 282 à 398 ou n° 298 à 398 de la formule (VI) indiquée dans la figure 4, ou la séquence des acides aminés n° 328 à 426 de la formule (VIII) indiquée dans la figure 4, ou qui est une protéine précurseur dont la séquence d'acides aminés comporte une séquence d'acides aminés représentée par l'une des formules (I), (II), (IV) et (V) indiquées dans la figure 3 ou par l'une des formules (VI) et (VIII) indiquées dans la figure 4, lequel procédé comporte le fait de cultiver un individu transformé non-humain qui héberge un ADN comportant un segment d'ADN codant ladite protéine, mais qui n'est pas un individu Xenopus laevis, le fait de laisser la protéine produite s'accumuler dans la culture, et le fait de recueillir la protéine ainsi obtenue.

2. Procédé de préparation d'un ADN comportant un segment d'ADN codant une protéine intervenant dans la morphogenèse des os de Xenopus laevis, définie dans la revendication 1, lequel procédé comporte le fait de cloner cet ADN à partir d'une bibliothèque d'ADN de Xenopus laevis.

3. Procédé de préparation d'un ADN comportant un segment d'ADN codant une protéine intervenant dans la morphogenèse des os de Xenopus laevis, dans lequel ledit segment d'ADN comporte une séquence de nucléotides qui correspond à une séquence de nucléotides représentée par l'une des formules (1), (2), (3), (4), (5), (6), (7) et (8) indiquées dans la figure 2, lequel procédé comporte le fait de cloner cet ADN à partir d'une bibliothèque d'ADN de Xenopus laevis.

4. Procédé de préparation d'un individu transformé non-humain qui héberge un ADN comportant un segment d'ADN, préparé conformément à l'une des revendications 2 et 3, l'individu transformé préparé n'étant pas un individu Xenopus laevis, lequel procédé comporte le fait de transformer un hôte avec ledit ADN.

5. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXar3 (FERM BP-2578).

6. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXar4 (FERM BP-2579).

7. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXar5 (FERM BP-2580).

8. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXar9 (FERM BP-2581).

9. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXar14 (FERM BP-2582).

10. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXbr22 (FERM BP-3066).

11. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXbr23 (FERM BP-3065).

12. Procédé conforme à la revendication 4, dans lequel l'individu transformé préparé présente les caractéristiques d'Escherichia coli HB101/pXbr41 (FERM BP-3067).

13. Procédé de préparation d'une composition destinée au traitement des fractures ou de l'ostéoporose, qui comporte le fait de mélanger une quantité efficace d'une protéine intervenant dans la morphogenèse des os de Xenopus laevis, préparée conformément à la revendication 1, avec des composants supplémentaires admissibles en pharmacie.

Fig. 1

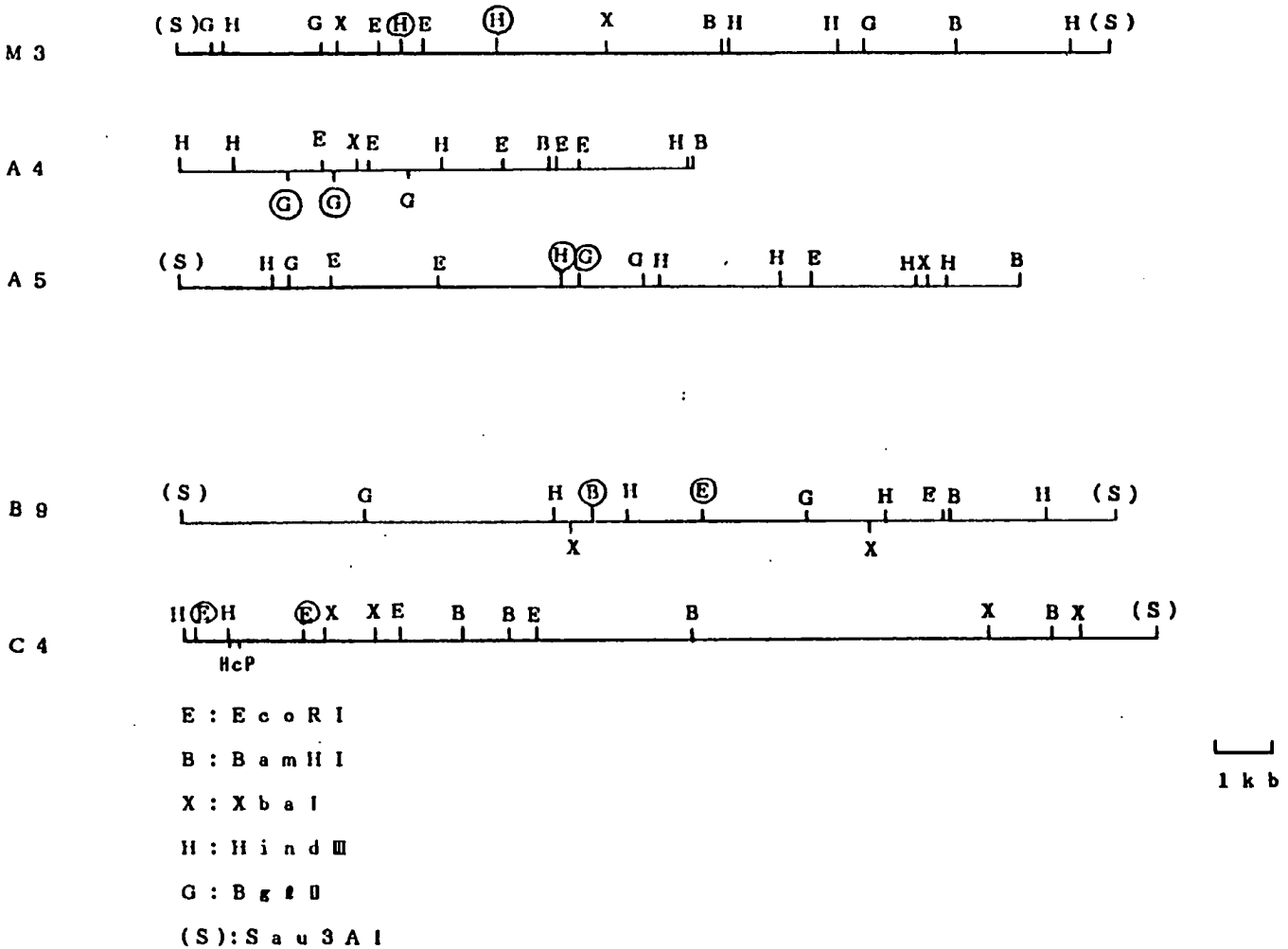
M 3
(S)G H G X E H E H X B H H G B H (S)
A 4
H H E X E H E B E E H B
G G G
A 5
(S) H G E E H G G H H E HX H B
B 9
(S) G H B H E G H E B H (S)
X X
C 4
H H H E X X E B B E B X B X (S)
HcP
E : E c o R I
B : B a m H I
X : X b a I
H : H i n d III
G : B g l II
(S): S a u 3 A I
1 k b

Fig. 2 (1)-1

B 9

Fig. 2 (1)-2

790 800 810 820 830 840
TAGCACCTCCTGGCTACCATGCCAATTATTGTGAGGGAGACTGCCCTAGCCATATTGCTG
AlaProProGlyTyrHisAlaAsnTyrCysGluGlyAspCysProSerHisIleAlaGly
850 860 870 880 890 900
GAACTACTGGCTCATCCTTGTCTTTTCACTCCACAGTTATAAACCAGTACAGACTAAGGG
ThrThrGlySerSerLeuSerPheHisSerThrValIleAsnGlnTyrArgLeuArgGly
910 920 930 940 950 960
GCCAAAGTCCCTTCACCAGCATCAAATCCTGCTGTGTGCCCTCTAAGCTTAGAGCTATGT
GlnSerProPheThrSerIleLysSerCysCysValProSerLysLeuArgAlaMetSer
970 980 990 1000 1010 1020
CCATGTTGTACTATGATGACGGTCAGAATATAATCAAAAAGGATATTCAAAATATGATTG
MetLeuTyrTyrAspAspGlyGlnAsnIleIleLysLysAspIleGlnAsnMetIleVal
1030 1040 1050 1060 1070 1080
TGGAGGAATGTGGCTGCTCATAAAGTTAAAAAGAGCATTGTCATTGTCAGCATTGCGTTG
GluGluCysGlyCysSer***
1090 1100 1110 1120 1130 1140
AAAATACACTAATCAAGAGAAGTGCAAATGAACAAAGATCAAAAGTCAAAGTTATCAGGG
1150 1160 1170 1180 1190 1200
TTTGGGTGGTGAACTTGATTCACAGTAAACAGCCTGCAGCAATTTTTATCCAAACGTGGA
1210 1220 1230 1240 1250 1260
AACCCCTTTTTAAGCACGACTGTGGATTTTATTTTAAATAAAAGCACAAGAACANATATA
1270 1280 1290 1300 1310 1320
AAGCTATTTATAAAATGTTTGCATATCTGACAGGTGCTTCTGTTACTGCACATGCGGGTA
1330 1340 1350 1360 1370 1380
TACANATGTGTATAAATAGGTACACTGTAAAGATATCCAGAGAGTCATGTTTGATGCCAT
1390 1400 1410 1420 1430 1440
GTTGTCAGTCAGCAGTTCATAGTTTCAGAAGATAAATACATTGCGCAGGTAATCTCACCT
1450 1460 1470 1480 1490 1500
TCTTCCTCTGCACTATCCTTGCAAAAGTATAAAAAAATACAGAAAAAAGGCAAACAAAGTT
1510 1520 1530 1540 1550 1560
AAATTTCACTACGGTGCTGATGATCATAGAACTATGCAACTTTTTGGGTTTGAAAACCGT
1570 1580 1590 1600 1610 1620
TTACCTGAGAATTAAAAGAGAGACTTTTACAGTGGAAGCAACGTAAATATTTTTGTTCTT
1630 1640 1650 1660
TCATGAGAGATACTTGAAAGGAACATGTTGGTCCAGGTTCTGGATCC

Fig. 2 (2)

M3

10 20 30 40 50 60
AAGCTTACAGTTGGAATTGAACTGTGATGGATGCCAAGATGTGCCAGTTTTAGCCAATCC
SerLeuGlnLeuGluLeuAsnCysAspGlyCysGlnAspValProValLeuAlaAsnPro
70 80 90 100 110 120
CAATAATTCCGACCAGCCATTTCTGGTAGCAGGCCAAAGTCGATGAACAAAGTCACCA
AsnAsnSerHisGlnProPheLeuValAlaGlnAlaLysValHisGluGlnSerHisHis
130 140 150 160 170 180
TGCTACAAAGAGAAGTCTTAACTGTGATCAGAACTCCAATCTGTGCTGTAGGAAAGACTA
AlaThrLysArgSerLeuAsnCysAspGlnAsnSerAsnLeuCysCysArgLysAspTyr
190 200 210 220 230 240
TTATGTAGACTTCAAGGATATTGGGTGGAATGATTGGATTATAAAGCCAGAGGGATATCA
TyrValAspPheLysAspIleGlyTrpAsnAspTrpIleIleLysProGluGlyTyrGln
250 260 270 280 290 300
GATAAATTATTGCATGGGCCTTTGCCCAATGCATATCCCTGGAGCCCCAGGTACGGGAGG
IleAsnTyrCysMetGlyLeuCysProMetHisIleAlaGlyAlaProGlyThrAlaAla
310 320 330 340 350 360
CTCATTCCACACCACGGTATTAAATCTCATTAAGGCCAACAATATCCAGACAGCAGTGAA
SerPheHisThrThrValLeuAsnLeuIleLysAlaAsnAsnIleGlnThrAlaValAsn
370 380 390 400 410 420
CTCATGCTGTGTCGCTACCAAAAGGCGCCCTTTGTCCATGCTTTACTTTGATAGAAATAA
SerCysCysValProThrLysArgArgProLeuSerMetLeuTyrPheAspArgAsnAsn
430 440 450 460 470 480
CAACGTTCTCAAGACTGACATTGCCGATATGATTGTGGAAGCCTGTGGGTGTAGCTAGGG
AsnValLeuLysThrAspIleAlaAspMetIleValGluAlaCysGlyCysSer***
490 500 510 520 530 540
CTTGGGCTACATCAGTTTGGGACATTTACAATAAAAAAGAGGGAAGCTGGCTTTTCTCTT
550 560 570 580 590 600
CATTATTTGCTCGAGATGCTTTAGACAGGTGAAGAACAAGTGAAAAAACTGATTCCACAT
610 620 630 640 650 660
TCACTTATCAAATTCATGGGAATCGTTAACANGATCANCCTCTCTAAACGGATCTAGGGA
670 680
GCTGTAGCACAATANCTNTCCAGCAGG

Fig. 2 (3)-1

C4

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        10          20          30          40          50          60
AGAGCAGGTCAAGAGCCCTTTGANAGTGACAGCAGCAAATTGCATCGGATTANTATTTAC

        70          80          90          100         110         120
GACATTGTCAGGSCAGCGGNNNNCTGGSCTSCCGGGGGCCTGTTGTGAGACTATTGGACA
                                  ArgGlyProValValArgLeuLeuAspThr

        130         140         150         160         170         180
CCAAACTGGTACATCATAATGAAAGCAAATGGGAAAGTTTTGATGTAGCGCCGGCAATTG
  LysLeuValHisHisAsnGluSerLysTrpGluSerPheAspValAlaProAlaIleAla

        190         200         210         220         230         240
CGCGGTGGATTGCACATAAACAGCCTAACCATGGGTTTGTTGTTGAAGTTACTCACTTGG
  ArgTrpIleAlaHisLysGlnProAsnHisGlyPheValValGluValThrHisLeuAsp

        250         260         270         280         290         300
ACAATGACAAAAATGTGCCTAAGAAGCATGTGAGGATTAGTAGGTCTTTAACCCCGGATA
  AsnAspLysAsnValProLysLysHisValArgIleSerArgSerLeuThrProAspLys

        310         320         330         340         350         360
AAGATAACTGGCCTCAGATACGGCCATTGTTGGTAACTTTTAGCCATGATGGTAAAGGAC
  AspAsnTrpProGlnIleArgProLeuLeuValThrPheSerHisAspGlyLysGlyHis

        370         380         390         400         410         420
ATGCTCTTCACAAAAGACAAAAGCGCCAAGCTAGGCACAAACAACGTAAACGCCTTAAAT
  AlaLeuHisLysArgGlnLysArgGlnAlaArgHisLysGlnArgLysArgLeuLysSer

        430         440         450         460         470         480
CGAGCTGCAGGAGGCATCCGTTGTACGTAGATTTCAGCGACGTTGGTTGGAATGACTGGA
  SerCysArgArgHisProLeuTyrValAspPheSerAspValGlyTrpAsnAspTrpIle

        490         500         510         520         530         540
TTGTTGCCCCACCTGGGTATCATGCCTTTTACTGCCACGGGGAATGTCCTTTTCCACTGG
  ValAlaProProGlyTyrHisAlaPheTyrCysHisGlyGluCysProPheProLeuAla
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Fig. 2 (3)-2

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         550         560         570         580         590         600
CAGACCATTTAAACTCTACAAACCATGCAATCGTACAAACTTTGGTGAACTCTGTCAACA
   AspHisLeuAsnSerThrAsnHisAlaIleValGlnThrLeuValAsnSerValAsnThr

         610         620         630         640         650         660
CAAACATCCCCAAAGCTTGCTGCGTCCCCACAGAACTCAGTGCCATATCCATGCTCTATC
   AsnIleProLysAlaCysCysValProThrGluLeuSerAlaIleSerMetLeuTyrLeu

         670         680         690         700         710         720
TTGATGAGAATGAAAAAGTAGTATTAAAAAATTATCAAGACATGGTCGTGGAGGGGTGCG
   AspGluAsnGluLysValValLeuLysAsnTyrGlnAspMetValValGluGlyCysGly

         730         740         750         760         770         780
GATGCCGTTAGGCAGTTACGCGCAAGCCAGAGACAAGAAAGATGACACTTTAATATTTCC
   CysArg***

         790         800         810         820         830         840
TTTTGGAGACTATATTTATGCTTTGAAAAATGATGAAACANTTATTTTGAAAATATATTT

         850         860         870         880         890         900
ATGTCTACACGGAGGTTGGGAAGCAAATATTTTAATCAGAGAAATATTCCTTTTTTTTAGT

         910         920         930         940         950
TGTACATTTTTATAAGGGTTTGTACCCAGCACATGAAGTATAATGGTCAGATTGA
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Fig. 2 (4)

A 4

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          10                  20                  30                  40                  50                  60
CCTGAGANTTAAGAAGTGTGGGATTTAACAGAACAGGACGACCGACCAATGAGAAAGCTA

          70                  80                  90                 100                 110                 120
TTTTTCTTGTCTNTGGTAGGACAAAGAAACGGGACNTGTTCTTCAATGAGATTAAAGCCA
                              PhePheAsnGluIleLysAlaArg

         130                 140                 150                 160                 170                 180
GGTCTGGCCAAGATGACAAGACTGTCTATGAATATTTATTCAATCAGAGGAGAAAGAGAC
   SerGlyGlnAspAspLysThrValTyrGluTyrLeuPheAsnGlnArgArgLysArgArg

         190                 200                 210                 220                 230                 240
GAGCTCCTCTGTCAACTAGGCAAGGGAAGAGGCCTAATAAGAATTCAAAAGCAAGATGTA
   AlaProLeuSerThrArgGlnGlyLysArgProAsnLysAsnSerLysAlaArgCysSer

         250                 260                 270                 280                 290                 300
GCAAGAAACCACTTCATGTCAATTTCAAGGATATGGGTTGGGATGATTGGATTATTGCCC
   LysLysProLeuHisValAsnPheLysAspMetGlyTrpAspAspTrpIleIleAlaPro

         310                 320                 330                 340                 350
CTTTGGAGTATGAGGCATATCATTGTGAAGGGCTTTGTGAGTTCCCTCTGAGATCT
   LeuGluTyrGluAlaTyrHisCysGluGlyLeuCysGluPheProLeuArgSer
```

Fig. 2 (5)

A 5

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          10                  20                  30                  40                  50                  60
AAGCTTTACTGGTGGTGTCTTCCCATTCCAAGAGGAGGGAAAACTTGTTTAAGGAGATCA

          70                  80                  90                 100                 110                 120
GGGACAAGATTAAGTCAATTGGAAATCCTAAATTCTTGGAGCCACCGGATTCAGTCAACA
                    IleLeuGlyAlaThrGlyPheSerGlnGln

         130                 140                 150                 160                 170                 180
GTCCATCGCCAAGAGGAGATGGAAACGAACAACTCTCCCCACTAGGACAAATAATGGCAA
  SerIleAlaLysArgArgTrpLysArgThrThrLeuProThrArgThrAsnAsnGlyLys

         190                 200                 210                 220                 230                 240
AGGTCATGCGAAGAAATCCAAAACAAGGTGTAGCAAGAAGCCCCTTCTTGTCAACTTCAA
  GlyHisAlaLysLysSerLysThrArgCysSerLysLysProLeuLeuValAsnPheLys

         250                 260                 270                 280                 290                 300
GGAGTTGGGTTGGGATGACTGGATTATTGCTCCCTTGGATTATGAAGCCTATCACTGCGA
  GluLeuGlyTrpAspAspTrpIleIleAlaProLeuAspTyrGluAlaTyrHisCysGlu

         310                 320
GGGGGTCTGTGATTTCCCACTGAGATCT
  GlyValCysAspPheProLeuArgSer
```

Fig. 2 (6)
pxbr22 (BMP2A)

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       10         20         30         40         50         60         70         80         90        100
GAATTCTCTTCCCTCTCACCGGCCTCTCGTCTCTACTCACCTCCCGGCGACCCCGGCTGGACTGAGACACTCGCTGCCACTATGTGCGACAACTCACCGA

      110        120        130        140        150        160        170        180        190        200
CTGGGCTCGACTGGACGCGCGGACTTGTCTCCCTCCTCTGGGGACCAGCGACTTGAACTAAAGACTCGAGTGATTGTGGAAAAAACACGCGGGGAGCAGA

      210        220        230        240        250        260        270        280        290        300
AAACCCACATCGAGACACAAACTCGGCGACTAAATCGCTCAGGTTGACAATGGTCGCTGGGATCCACTCTCTGCTCCTGCTGCAGTTTTACCAGATCTTG
                                                  M  V  A  G  I  H  S  L  L  L  L  Q  F  Y  Q  I  L

      310        320        330        340        350        360        370        380        390        400
CTGAGCGGCTGCACCGGGCTCGTCCCAGAGGAAGGCAAACGCAAGTATTCCGAATCCACTCGCTCGTCTCCGCAGCAGTCCCAACAAGTCCTCGACCAGT
L  S  G  C  T  G  L  V  P  E  E  G  K  R  K  Y  S  E  S  T  R  S  S  P  Q  Q  S  Q  Q  V  L  D  Q  F

      410        420        430        440        450        460        470        480        490        500
TTGAGCTTCGGCTGCTCAATATGTTCGGCTTGAAGAGGAGGCCGACGCCTGGCAAAAATGTTGTGATCCCCCCCTACATGTTGGACTTGTACCACCTGCA
  E  L  R  L  L  N  M  F  G  L  K  R  R  P  T  P  G  K  N  V  V  I  P  P  Y  M  L  D  L  Y  H  L  H

      510        520        530        540        550        560        570        580        590        600
CTCGGCTCAGTTGGCCGATGATCAAGGAAGTTCTGAGGTGGACTATCACATGGAGCGGGCGGCTAGCAGAGCCAACACAGTGAGGAGCTTTCACCATGAA
 S  A  Q  L  A  D  D  Q  G  S  S  E  V  D  Y  H  M  E  R  A  A  S  R  A  N  T  V  R  S  F  H  H  E

      610        620        630        640        650        660        670        680        690        700
GAATCCATGGAAGAAATTCCAGAGTCTGGTGAGAAAACAATCCAACGATTCTTCTTCAACCTTTCTTCAATTCCAGATGAGGAGCTGGTCACGTCTTCTG
E  S  M  E  E  I  P  E  S  G  E  K  T  I  Q  R  F  F  F  N  L  S  S  I  P  D  E  E  L  V  T  S  S  E

      710        720        730        740        750        760        770        780        790        800
AGCTCCGGATTTTTCGAGAGCAGGTCCAAGAGCCATTTAAGACTGACGGCAGCAAACTTCATCGGATTAATATTTATGACATTGTCAAGCCAGCGGCGGC
  L  R  I  F  R  E  Q  V  Q  E  P  F  K  T  D  G  S  K  L  H  R  I  N  I  Y  D  I  V  K  P  A  A  A

      810        820        830        840        850        860        870        880        890        900
TGCCTCCCGGGGCCCTGTTGTAAGACTATTGGACACCAGACTGATCCATCATAATGAAAGCAAATGGGAAAGTTTTGATGTGACGCCGGCAATTACACGG
 A  S  R  G  P  V  V  R  L  L  D  T  R  L  I  H  H  N  E  S  K  W  E  S  F  D  V  T  P  A  I  T  R

      910        920        930        940        950        960        970        980        990       1000
TGGATTGCACATAAACAGCCTAACCATGGGTTTGTTGTTGAAGTGACTCACTTGGACAATGACACAAATGTGCCCAAGAGGCATGTGAGGATTAGTAGGT
W  I  A  H  K  Q  P  N  H  G  F  V  V  E  V  T  H  L  D  N  D  T  N  V  P  K  R  H  V  R  I  S  R  S

      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
CTTTAACCCTGGATAAAGGTCACTGGCCTCGGATACGGCCATTATTGGTAACTTTTAGCCATGATGGCAAAGGACATGCTCTTCACAAAAGACAAAAACG
  L  T  L  D  K  G  H  W  P  R  I  R  P  L  L  V  T  F  S  H  D  G  K  G  H  A  L  H  K  R  Q  K  R

      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
GCAAGCTAGGCACAAACAACGTAAACGCCTTAAATCGAGCTGCAGGAGGCATCCGTTGTACGTAGATTTCAGTGACGTTGGTTGGAATGACTGGATTGTT
 Q  A  R  H  K  Q  R  K  R  L  K  S  S  C  R  R  H  P  L  Y  V  D  F  S  D  V  G  W  N  D  W  I  V

      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
GCCCCACCTGGGTATCATGCCTTTTACTGCCACGGGGAATGTCCTTTTCCACTGGCAGACCATTTAAACTCTACAAACCATGCAATCGTACAAACTTTGG
A  P  P  G  Y  H  A  F  Y  C  H  G  E  C  P  F  P  L  A  D  H  L  N  S  T  N  H  A  I  V  Q  T  L  V

      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
TGAATTCCGTCAACACAAACATTCCCAAAGCTTGCTGCGTCCCCACAGAACTCAGTGCCATCTCCATGCTCTATCTTGATGAGAATGAAAAAGTAGTATT
  N  S  V  N  T  N  I  P  K  A  C  C  V  P  T  E  L  S  A  I  S  M  L  Y  L  D  E  N  E  K  V  V  L

      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
AAAGAATTATCAAGACATGGTCGTGGAGGGGTGCGGGTGCCGTTAGGCGGGGACACACAAGCCAGAGACAAGAAAGCTGACACTTTAATATTTCCTTTTG
K  N  Y  Q  D  M  V  V  E  G  C  G  C  R  *

      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
GAGACTATATTTATGCTTTGAAAAATGATGAAACAATTATTTTGAAAATATATTTATGTCTACACGGAGGCTGGGAAGCAAATATTTTAATCAGAGAAAT

      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
ATTCCTTTTTAGTTGTACATTTTTATAAGGGTTTGTACCCAGCACATGAAGTATAATGGTCAGATTCCTATTTTGTATTTATTTACCATTATAACCACTT

      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
TTTAAGGAAAAAAAATAGCTGTTTTGTATTTATATGTAATCAACAGAGAAAATATAGGGTTTGTAAATATGTTACTGAAAGTGTTTTTTTCTTCTTTTTTT

      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
TAAATTATGTATACACAGCTGGTTATATGGCAAGTTTTTTATATTTTCTATAAAGCTAATTTCAAGGTCATTAGTTATAAACTTGATGATGTGTTGGTTC

      1910       1920       1930       1940       1950       1960       1970       1980       1990
ATTGGTAAATCCTCCATATTGTGCAATTAACATGCATTTTTATAATGTACGAAGTCCAGTCCATTGTGCATTGCTTTGCAAATTTAGAATTC
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Fig. 2. (7)
pxbr23 (BMP2B)

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      10        20        30        40        50        60        70        80        90       100
GGAATTCCGGCCCCACTGAGCTTTTCCACACATTTTTTGTGTCCAACATTGGCTGTCAAGAATCATGGAATGTTTTTCTATGCCTTGTTTTCTGTCAAGA

     110       120       130       140       150       160       170       180       190       200
CATCATGATTCCTGGTAACCGAATGCTGATGGTCATTTTATTAAGCCAAGTCCTGCTCGGAGGCACTAACTATGCCAGCCTGATACCTGACACGGGCAAG
   M  I  P  G  N  R  M  L  M  V  I  L  L  S  Q  V  L  L  G  G  T  N  Y  A  S  L  I  P  D  T  G  K

     210       220       230       240       250       260       270       280       290       300
AAGAAAGTCGCGGCCGACATTCAGGGAGGAGGTCGCAGGTCGCCTCAGAGCAATGAGCTCTTGCGGGATTTCGAGGTGACGCTGCTGCAGATGTTCGGAC
K  K  V  A  A  D  I  Q  G  G  G  R  R  S  P  Q  S  N  E  L  L  R  D  F  E  V  T  L  L  Q  M  F  G  L

     310       320       330       340       350       360       370       380       390       400
TCCGCAAGCGGCCGCAGCCCAGTAAGGATGTGGTGGTTCCCGCTTATATGCGCGACCTGTACAGGCTTCAGTCAGCGGAGGAGGAGGATGAACTGCACGA
  R  K  R  P  Q  P  S  K  D  V  V  V  P  A  Y  M  R  D  L  Y  R  L  Q  S  A  E  E  E  D  E  L  H  D

     410       420       430       440       450       460       470       480       490       500
TATCAGCATGGAGTACCCCGAGACACCCACCAGCCGCGCCAACACCGTGAGGAGCTTCCATCACGAGGAACATTTGGAGAATCTACCAGGCACAGAAGAA
 I  S  M  E  Y  P  E  T  P  T  S  R  A  N  T  V  R  S  F  H  H  E  E  H  L  E  N  L  P  G  T  E  E

     510       520       530       540       550       560       570       580       590       600
AATGGAAATTTCCGTTTTGTGTTCAACCTCAGCAGCATTCCAGAGAATGAGGTGATTTCTTCAGCAGAACTGAGACTCTATAGAGAACAAATAGACCATG
N  G  N  F  R  F  V  F  N  L  S  S  I  P  E  N  E  V  I  S  S  A  E  L  R  L  Y  R  E  Q  I  D  H  G

     610       620       630       640       650       660       670       680       690       700
GTCCAGCGTGGGATGAGGGTTTCCACCGGATAAATATATATGAAGTTATGAAACCCATCACAGCAAACGGACACATGATAAATAGGCTGCTGGACACGAG
  P  A  W  D  E  G  F  H  R  I  N  I  Y  E  V  M  K  P  I  T  A  N  G  H  M  I  N  R  L  L  D  T  R

     710       720       730       740       750       760       770       780       790       800
GGTAATCCACCACAATGTGACACAGTGGGAAAGTTTTGATGTAAGCCCTGCAATTATGAGGTGGACCCTGGATAAACAGATAAACCATGGGCTTGCCATT
 V  I  H  H  N  V  T  Q  W  E  S  F  D  V  S  P  A  I  M  R  W  T  L  D  K  Q  I  N  H  G  L  A  I

     810       820       830       840       850       860       870       880       890       900
GAGGTCATTCACCTCAACCAAACAAAAACTTATCAGGGGAAGCATGTAAGGATAAGTCGATCTTTATTACCTCAAAAGGATGCAGACTGGTCACAGATGA
E  V  I  H  L  N  Q  T  K  T  Y  Q  G  K  H  V  R  I  S  R  S  L  L  P  Q  K  D  A  D  W  S  Q  M  R

     910       920       930       940       950       960       970       980       990      1000
GACCACTTTTAATTACATTCAGCCATGATGGCAGGGGGCATGCACTGACTAGGAGGTCAAAAAGAAGTCCAAAACAGCAGAGACCCCGTAAAAAAAATAA
  P  L  L  I  T  F  S  H  D  G  R  G  H  A  L  T  R  R  S  K  R  S  P  K  Q  Q  R  P  R  K  K  N  K

    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
ACACTGCCGGAGACATTCTCTTTATGTGGATTTCAGCGATGTGGGCTGGAATGATTGGATTGTGGCACCTCCTGGATACCAGGCCTTTTACTGCCATGGA
 H  C  R  R  H  S  L  Y  V  D  F  S  D  V  G  W  N  D  W  I  V  A  P  P  G  Y  Q  A  F  Y  C  H  G

    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
GATTGTCCATTTCCCTTGGCTGATCACCTAAACTCAACTAACCATGCTATTGTACAAACTCTGGTAAACTCTGTTAACTCAAGCATCCCAAAAGCATGCT
D  C  P  F  P  L  A  D  H  L  N  S  T  N  H  A  I  V  Q  T  L  V  N  S  V  N  S  S  I  P  K  A  C  C

    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
GCGTCCCCACAGAACTGAGTGCTATCTCCATGCTTTATTTGGATGAATATGACAAAGTCGTCCTTAAAAACTACCAGGAGATGGTGGTGGAAGGGTGTGG
 V  P  T  E  L  S  A  I  S  M  L  Y  L  D  E  Y  D  K  V  V  L  K  N  Y  Q  E  M  V  V  E  G  C  G

    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
GTGCCGTTGAGTCTGAGATCCAAACAAAAGACTGTTAACGGCTGGACTTCTTTCCACTGAACATTCACCTTGACCTTATTTATGACTTTTATGTGTAAAT
 C  R  *

    1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
GTTTTTTTGACAATATGATCATATATTTTGACAAAATATATTTATAACTACGTATTAAAAGAAAAAAAAAAAAATAAAATAAGTCATTATTTTAAACATAA

    1510      1520      1530      1540      1550
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACGGAATTC
```

Fig. 2 (8)
pxbr41 (Vgrl)

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      10        20        30        40        50        60        70        80        90       100
GAATTCCGATATGGAATGTAAAAATACTGGTGAATTATGGGAAGTCCGACACAGACCACTAACTTCAGCATCTTATCTTTGACAAAATGAATGCTTTGAC
                                                                                    M  N  A  L  T

     110       120       130       140       150       160       170       180       190       200
AGTAAAGAGAAGATTGCCTGTGCTGCTTTTTCTTTTTCACATTTCACTGAGTTCCATCTCGTCAAATACAATATTGGAGAATGATTTCCACTCTAGTTTT
 V  K  R  R  L  P  V  L  L  F  L  F  H  I  S  L  S  S  I  S  S  N  T  I  L  E  N  D  F  H  S  S  F

     210       220       230       240       250       260       270       280       290       300
GTCCAGAGAAGACTAAAAGGCCACGAACGCAGAGAGATTCAAAAAGAGATCTTGACTATTTTAGGTTTGCAACACAGACCAAGGCCATATTTACCGGAGA
 V  Q  R  R  L  K  G  H  E  R  R  E  I  Q  K  E  I  L  T  I  L  G  L  Q  H  R  P  R  P  Y  L  P  E  K

     310       320       330       340       350       360       370       380       390       400
AAAAGAAGTCTGCACCATTATTCATGATGGATTTATACAATGCAGTAAATATTGAAGAGATGCATGCTGAAGATGTTTCCTACAGCAATAAGCCGATCTC
  K  K  S  A  P  L  F  M  M  D  L  Y  N  A  V  N  I  E  E  M  H  A  E  D  V  S  Y  S  N  K  P  I  S

     410       420       430       440       450       460       470       480       490       500
CCTAAATGAAGCTTTTTCACTGGCCACTGACCAAGAGAATGGCTTTCTTGCACATGCCGACACAGTTATGAGTTTTGCTAATTTAGTTGACAATGACAAC
 L  N  E  A  F  S  L  A  T  D  Q  E  N  G  F  L  A  H  A  D  T  V  M  S  F  A  N  L  V  D  N  D  N

     510       520       530       540       550       560       570       580       590       600
GAATTGCATAAAAACTCCTATCGCCAAAAATTCAAGTTTGATCTAACTGATATCCCACTTGGAGATGAACTGACAGCCGCTGAATTTCGAATTTATAAAG
E  L  H  K  N  S  Y  R  Q  K  F  K  F  D  L  T  D  I  P  L  G  D  E  L  T  A  A  E  F  R  I  Y  K  D

     610       620       630       640       650       660       670       680       690       700
ATTATGTACAAAATAACGAGACATACCAGGTCACCATCTACCAGGTGCTTAAGAAGCAAGCCGACAAAGATCCTTATCTTTTCCAGGTAGACTCAAGAAC
  Y  V  Q  N  N  E  T  Y  Q  V  T  I  Y  Q  V  L  K  K  Q  A  D  K  D  P  Y  L  F  Q  V  D  S  R  T

     710       720       730       740       750       760       770       780       790       800
CATCTGGGGCACAGAAAAGGGATGGCTGACGTTTGATATTACTGCAACTGGTAATCACTGGGTGATGAACCCACATTACAACCTTGGATTGCAGTTATCA
 I  W  G  T  E  K  G  W  L  T  F  D  I  T  A  T  G  N  H  W  V  M  N  P  H  Y  N  L  G  L  Q  L  S

     810       820       830       840       850       860       870       880       890       900
GTAGAGAGTATGGATATGCAAAATGTTAATCCCAGGCTTGTGGGCCTTGTTGGAAAGAATGGTCCTCAAGACAAACAGCCATTTATGGTGGCATTCTTTA
V  E  S  M  D  M  Q  N  V  N  P  R  L  V  G  L  V  G  K  N  G  P  Q  D  K  Q  P  F  M  V  A  F  F  K

     910       920       930       940       950       960       970       980       990      1000
AGACCTCAGATATCCATCTCCGCAGTGTTCGATCTACTAGCAATAAGCACTGGAATCAGGAAAGAGCCAAGACCTACAAGGAGCAAGATAATTTACCTCC
  T  S  D  I  H  L  R  S  V  R  S  T  S  N  K  H  W  N  Q  E  R  A  K  T  Y  K  E  Q  D  N  L  P  P

    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
AGCAAATATTACTGATGGCATCATGCCCCCTGGAAAACGTCGTTTTTTAAAGCAAGCTTGCAAGAAACATGAACTGTTTGTAAGTTTCCGCGATCTTGGT
 A  N  I  T  D  G  I  M  P  P  G  K  R  R  F  L  K  Q  A  C  K  K  H  E  L  F  V  S  F  R  D  L  G

    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
TGGCAAGACTGGATAATTGCACCTGAAGGATATGCTGCCTACTATTGTGATGGAGAATGTGCTTTCCCACTTAACTCTTTCATGAATGCCACAAACCATG
W  Q  D  W  I  I  A  P  E  G  Y  A  A  Y  Y  C  D  G  E  C  A  F  P  L  N  S  F  M  N  A  T  N  H  A

    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
CCATTGTACAAACGTTGGTACATTTCATTAACCCAGAGACTGTCCCTAAGCCATGCTGTGCACCAACTCAGCTCAATGGTATTTCTGTTTTTATACTTTGA
  I  V  Q  T  L  V  H  F  I  N  P  E  T  V  P  K  P  C  C  A  P  T  Q  L  N  G  I  S  V  L  Y  F  D

    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
TGACAGTGCCAATGTTATATTAAAGAAATACAAAAATATGGTGGTTCAAGCCTGTGGTTGCCATTGACAATAGCAGTTATTCTGTTTTTAACAGTCATTT
 D  S  A  N  V  I  L  K  K  Y  K  N  M  V  V  Q  A  C  G  C  H  *

    1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
TAATGGTATTGTCCTTATCGTTTATTTTAAAGTAGAGATACTTGACCATCACACTTAAAAAAATGCATTGTACACCTTAACGGATGAAAAGATTTTGTTT

    1510
TTGCATGATTTCGGAATTC
```

Fig. 3

```
βA          ARQSEDHPHRRRRRR--GLECDGKVNICCKKQFFVSFKDIGWNDWIIAPSGYHANYCEGECPSHIAG
B9(I)       . . . TDE. . . . . KK. --. . . . . S. . . . . H. Y. . . . . S. . . . . P. . . . . D. . . . . .
M3(II)      AK-VHEQS. HATK. --S. N. . QNS. L. . R. DYY. D. . . . . K. E. . QI. . . M. L. . M. . . .
βB          . . LGDSR-. . I. K. --. . . . . RTSL. . RQ. . . ID. RL. . . . . T. . YG. . . . . S. . AYL. .
BMP2A       K. EKRQAK. KQ. K. ---. -----. SS-. KRHPLY. D. S. V. . . . . V. . P. . . . F. . H. . . . FPL. D
C4(III)     K. . KRQAR. KQ. K. ---. -----. SS-. RRHPLY. D. S. V. . . . . V. . P. . . . F. . H. . . . FPL. D
DPPC        . . . -HARRPT. . KN---H-----DDT-. RRHSLY. D. S. V. . DD. . V. . L. . D. Y. . H. K. . FPL. D
Vg1         TLNPLRCKRP. . K. SYSKLPFTAS. . . K. RHLY. E. . . V. . QN. V. . . Q. . M. . . . Y. . . . YPLTE
A4(IV)      R. KRRAPLST. --QGKRPNKNS. AR-. S. . PLH. N. . . M. . D. . . . . LE. E. YH. . . L. EFPLRS
A5(V)       R. WKRTTLPT. TNNGK. HAKKS. TR-. S. . PLL. N. . EL. . D. . . . . LD. E. YH. . . V. DFPLRS
CONSENSUSE  . . . . . C. . . . . F. . . GW. . W. . . P. . Y. . . . C. G. C. . . . . .

βA          TSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKLRPMSMLYYDDGQNIIKKDIQNMIVEECGCS
B9(I)       . T. . . . . Q. . L. . Q. . . TSI. . . . . S. . . A. . . . . .
M3(II)      AP. TAA. . . T. . L. LIK--ANNIQTAVN. . . . . R. . L. . . . F. RNN. VL. T. . AD. . . . A. . . .
βB          VP. . AS. . . TA. V. Q. . . . . LN. -GPVN. . . I. . . . SS. . . . . F. . EY. . V. R. VP. . . . . A
BMP2A       H--LNSTN. AI. QTLVN--SVNSK-IP. A. . . . . E. SAI. . . . L. ENEKVVL. NY. D. V. . G. . . R
C4(III)     H--LNSTN. AI. QTLVN--SVNTN-IP. A. . . . . E. SAI. . . . L. ENEKVVL. NY. D. V. . G. . . R
DPPC        H--FNSTN. AV. QTLVN-NMNPGK-VP. A. . . . . QLDSVA. . . LN. QSTVVL. NY. E. T. VG. . . R
Vg1         I--LNG. N. AILQTLVH--SIE. EDIPLP. . . . . MS. I. . . F. . NND. VVLRHYE. . A. D. . . . R
                ***
CONSENSUS   . . . . . H. . . . . CC. P. . . . . ML. . D. . . . . M. V. . CGC.
```

Fig. 4-1

(VI) BMP2A

M V A G I H S L L L L Q F Y Q I L

L S G C T G L V P E E G K R K Y S E S T R S S P Q Q S Q Q V L D Q F

E L R L L N M F G L K R R P T P G K N V V I P P Y M L D L Y H L H

S A Q L A D D Q G S S E V D Y H M E R A A S R A N T V R S F H H E

E S M E E I P E S G E K T I Q R F F F N L S S I P D E E L V T S S E

L R I F R E Q V Q E P F K T D G S K L H R I N I Y D I V K P A A A

A S R G P V V R L L D T R L I H H N E S K W E S F D V T P A I T R

W I A H K Q P N H G F V V E V T H L D N D T N V P K R H V R I S R S

L T L D K G H W P R I R P L L V T F S H D G K G H A L H K R Q K R

Q A R H K Q R K R L K S S C R R H P L Y V D F S D V G W N D W I V

A P P G Y H A F Y C H G E C P F P L A D H L N S T N H A I V Q T L V

N S V N T N I P K A C C V P T E L S A I S M L Y L D E N E K V V L

K N Y Q D M V V E G C G C R *

Fig. 4-2

(VII) BMP2B

M I P G N R M L M V I L L S Q V L L G G T N Y A S L I P D T G K

K K V A A D I Q G G G R R S P Q S N E L L R D F E V T L L Q M F G L

R K R P Q P S K D V V V P A Y M R D L Y R L Q S A E E E D E L H D

I S M E Y P E T P T S R A N T V R S F H H E E H L E N L P G T E E

N G N F R F V F N L S S I P E N E V I S S A E L R L Y R E Q I D H G

P A W D E G F H R I N I Y E V M K P I T A N G H M I N R L L D T R

V I H H N V T Q W E S F D V S P A I M R W T L D K Q I N H G L A I

E V I H L N Q T K T Y Q G K H V R I S R S L L P Q K D A D W S Q M R

P L L I T F S H D G R G H A L T R R S K R S P K Q Q R P R K K N K

H C R R H S L Y V D F S D V G W N D W I V A P P G Y Q A F Y C H G

D C P F P L A D H L N S T N H A I V Q T L V N S V N S S I P K A C C

V P T E L S A I S M L Y L D E Y D K V V L K N Y Q E M V V E G C G

C R *

Fig.4-5

(VH) (Vgrl)

M N A L T

V K R R L P V L L F L F H I S L S S I S S N T I L E N D F H S S F

V Q R R L K G H E R R E I Q K E I L T I L G L Q H R P R P Y L P E K

K K S A P L F M M D L Y N A V N I E E M H A E D V S Y S N K P I S

L N E A F S L A T D Q E N G F L A H A D T V M S F A N L V D N D N

E L H K N S Y R Q K F K F D L T D I P L G D E L T A A E F R I Y K D

Y V Q N N E T Y Q V T I Y Q V L K K Q A D K D P Y L F Q V D S R T

I W G T E K G W L T F D I T A T G N H W V M N P H Y N L G L Q L S

V E S M D M Q N V N P R L V G L V G K N G P Q D K Q P F M V A F F K

T S D I H L R S V R S T S N K H W N Q E R A K I Y K E Q D N L P P

A N I T D G I M P P G K R R F L K Q A C K K H E L F V S F R D L G

W Q D W I I A P E G Y A A Y Y C D G E C A F P L N S F M N A T N H A

I V Q T L V H F I N P E T V P K P C C A P T Q L N G I S V L Y F D

D S A N V I L K K Y K N M V V Q A C G C H *

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